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STUDIES OF THE FREE AND BOUND AMINO ACIDS IN HEALTHY AND DISEASED WHEAT ROOTS

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April, 1952

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T H E U N I V E R S I T Y O F A L B E R T A

STUDIES OF THE FREE AND BOUND AMINO ACIDS IN HEALTHY AND DISEASED WHEAT ROOTS

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
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FACULTY OF ARTS AND SCIENCE

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ABSTRACT

The technique of paper chromatography has been adapted to the identification of the free and bound amino acids of healthy and diseased wheat roots of the Thatcher variety. N-butanol, glacial acetic acid and water (4:1:5 by volume), and phenol-water (80% phenol by volume) was found to be a satisfactory solvent pair for two dimensional studies. A total of 13 known amino acids and six unknown ninhydrin spots were found in alcoholic extracts of dried wheat root samples. Chromatograms showed a lower concentration of these amino acids, except glutamic acid, in healthy than in those infected with the root-rot organism Helminthosporium sativum. Kjeldahl determinations also showed a lower total nitrogen content in the healthy roots. Certain free amino acids added to Czapek's medium on which H. sativum was subsequently cultured were found to decrease the pathogenicity of this fungus to wheat seedlings. The evidence suggests that certain amino acids may be of importance in the relative susceptibility of different varieties to H. sativum.

STUDIES OF THE FREE AND BOUND AMINO ACIDS IN HEALTHY AND DISEASED WHEAT ROOTS

S. B. Hrushovetz

INTRODUCTION

Paper chromatographic analysis is a very recent development in analytical chemistry. Consden, Gordon, and Martin (41) first used paper strips for the separation of amino acids in 1944. In less than ten years almost every group of compounds has been investigated and separated by partition chromatography procedures on paper.

This dissertation has a threefold purpose:

(a) to review the literature on paper chromatography;

(b) to apply these techniques to an investigation of a problem in plant pathology; and finally,

(c) to interpret the results of these investigations.

P A R T I

THEORY AND PRACTICE OF THE TECHNIQUES OF PAPER PARTITION CHROMATOGRAPHY

PART I

THEORY AND PRACTICE OF THE TECHNIQUES OF PAPER PARTITION CHROMATOGRAPHY

INTRODUCTION

The literature on chromatography, especially in the last five years, has been very extensive. As a result only the more important references will be cited. Chromatography, as any branch of analytical chemistry, has developed a terminology all its own. To facilitate a clearer understanding of the discussion to follow, definitions of the various terms and symbols that may be encountered in the literature are therefore included here.

GLOSSARY

Adsorbent - is the bulk phase consisting of either solid or liquid at the boundary of which the adsorption occurs (34).

Adsorption - accumulation of a solute or substance in a boundary region. It is a phenomenon of the interphase and the requirement for adsorption is that the concentration of the substance in the interphase be greater than that of the substance in the contiguous phases. (34). Adsorption depends on the

preferential affinity of the adsorbent for certain structural groups of the solute molecules (169).

Adsorptive - substance that is adsorbed.

Aqueous phase - in mixing two partially miscible liquids, one being water, so that two phases appear, the one containing the higher concentration of water is the aqueous phase (41).

Ascending technique - upward or vertical migration of the solvent or mobile phase on a strip or sheet of filter paper (188).

Bioautograph - a developed chromatogram in which the different solutes are revealed microbiologically (191).

Capillary analysis - method of chemical analysis introduced by Schoenbein and Goppelsroeder in which the substances separate into distinct zones due to capillary action. The principle here is similar to Tswett's chromatographic adsorption (170).

Chamber - an air-tight vessel employed for irrigating strips or sheets of filter paper with a solvent (41).

Chromatobar - a casting of the adsorbent of a chromatostrip (114).

Chromatogram - a developed or irrigated sheet of filter paper containing the solute mixture. Also see page nine.

Chromatopak - a column prepared by placing sheets or strips of filter paper side by side (114, 118). It is similar to chromatopile.

Chromatography - method of organic analysis introduced by Tswett. It depends on different degrees of adsorption of compounds or solutes in the same and different adsorbents and solvents. (200).

Chromatopile - column prepared by clamping together a large number of filter papers (114).

Chromatostrip - a glass slide coated with a film of the adsorbent (114).

Column - glass tubing filled with an adsorbent. This is used for adsorption or chromatographic analysis.

Countercurrent distribution - a discontinuous extraction process carried to a high number of stages(46). It is actually a partition effect where two immiscible liquids are allowed to flow past each other and the solute present distributes itself between the two liquids.

Descending technique - downward migration of solvent or mobile phase on a strip or sheet of filter paper in a chamber carrying the solute mixture with it (41).

Developer - is the solvent or mobile phase that is used to irrigate the paper and <u>does not</u> refer to the solution used for spraying the chromatogram.

Displacement development - where the original mixed solution is placed on the column in small volume and is washed through with a solution of some displacing agent (171).

Elution development - where the original mixed solution is placed on the column in small volume and washed through with pure solvent (171).

Frontal analysis - where the original mixed solution is passed continuously through the column (171).

Ion exchange - the replacing of the surface ions of a resin or other similar substances in a column, by the solutes being investigated (96).

Ionography - the separation of substances with small differences in ionic mobility at given pH's by use of a current and employing a slab of silica jelly (42) or filter paper (107) instead of a diaphragm cell.

Liquid chromatogram - where portions of a liquid percolating through a column are collected separately, e.g., the automatic sampling collectors of Moore and Stein (119).

Mobile phase - is that phase in a chromatographic system that travels carrying the solutes with it. Actually the solutes are in the interphase between the mobile and stationary phase.

Papryography - the name suggested by Dent (53) for partition chromatography on paper.

Partition - often called adsorption and denotes the amount of solute in the two phases (46).

Partition coefficient - see Symbols.

Partography - a name often applied to the type of partition chromatography introduced by Martin and Synge (112, 146) where they immobilized one of the liquid phases, i.e., papryography.

Solute - the known or unknown substance that is spotted on the paper.

Solvent - is the liquid used to irrigate the filter paper and is often called the mobile phase.

Spraying reagent - is a substance sprayed on a dried chromatogram to show various constituents. Work is related to Fiegl's spot test technique (58).

Stationary phase - or immobile phase. In paper chromatography the aqueous phase of the solvent together with the filter paper make up the stationary phase.

Surface active - same as Adsorption. See above.

Theoretical plates - refers to the number of separations that occur in partition chromatography. A certain definite number of plates or separations are required before one can get separation of two similar solutes in a mixture.

Ultrachromatography - application of ultra violet light to detect zones in developed columns or spots on paper chromatograms (200).

SYMBOLS

A - cross sectional area of paper, plus water, plus solvent (111).

A_L - cross sectional area of solvent phase (111).

As - cross sectional area of water phase (111).

α - partition coefficient =

$$\frac{\text{grams solute/ml. of non mobile phase}}{\text{grams solute/ml. of mobile phase}} \quad (112)$$

R - $\frac{\text{movement of position of maximum conc. of solute}}{\text{simultaneous movement of surface of developing phase in empty part of tube above chromatographic column}}$ (111).

R_f - $\frac{\text{movement of solute}}{\text{movement of advancing front of liquid}}$ (111).

R_m - a term introduced by Martin which is used in explaining thermodynamically relation of α and R_f. (18).

Relation: $R_m = \log \left(\frac{1}{R_f} - 1\right)$

R_x - relative position obtained by a solute after the solvent front has been allowed to run off the end of the paper. An amino acid like alanine is taken as unity and the position of the others are determined from it (55).

R_G - distance travelled by acetyl sugars and sugar derivatives taking tetra-acetyl-glucose as unity (24).

HISTORY OF CHROMATOGRAPHY

The best review on this topic is given by Zechmeister and Cholnoky (200). In their discussion of priority they give Michael Tswett most of the credit. The first eight page article that this botanist published in June 1906, which was on the chromatography of the leaf pigments of plants using powdered chalk as the adsorbent and petrol ether as the solvent, is taken by the authors as the official birthday of chromatography. This may be true for adsorption chromatography in a column, although Weil and Williams (185) do not agree. However, paper chromatography actually originated in 1861 when Schonbein and his pupil Goppelsroeder observed that solutes of dyes concentrate in distinct zones if solutions of them are drawn into strips of filter paper by capillary action (170).

Partition of organic solutes between two immiscible liquids, i.e., counter current distribution (46), has been widely used in organic chemistry for extraction purposes. However, with this technique many difficulties are encountered, the chief one being that the process is discontinuous and so makes extraction of an homologous series very tedious. Martin and Synge (112) overcame this difficulty by partition in a column. They immobilized one of the liquids (water) by soaking silica in water until a gel formed, and this silica-water complex was placed in a glass cylinder and formed the

immobile liquid phase. Thus they obtained a continuous extraction process using Craig's partition or counter current distribution principle. This column has little or no adsorption properties since the silica serves only as mechanical support for the water. Three years elapsed (1944) before the ingenious idea of employing filter paper as the mechanical support for the stationary liquid phase was tried. Because of this discovery, Consden, Gordon and Martin (41) are recognized as the pioneers in paper partition chromatography. Probably the use of starch and powdered cellulose in Tswett's columns as adsorbents might have been partially responsible for this very useful new development in analysis. The method of cross capillary analysis or two dimensional chromatography (41, 53) quickly followed. To this were soon added reverse phase techniques (192, 127), impregnation of paper with adsorbents (49), electrophoretic adaptations (107), salting out processes (103) and many other valuable modifications. The result is that very many groups of compounds in both the organic and inorganic field, as shown in Table 2, have been analyzed and separated by the careful choice of solvents and the use of the modifications listed above.

TYPES OF CHROMATOGRAPHY

Tswett was the originator of the term Chromatography. In discussing a petroleum ether solution extract of chlorophyll pigments after they were placed in a column and

washed with solvent, Tswett writes, ".....then the pigments according to the adsorption sequence, are resolved from top to bottom in colored zones, since the stronger adsorbed pigments displace the weaker adsorbed ones and force them downwards .... Like light rays in the spectrum, so the different components of a pigment mixture are resolved on the calcium carbonate column according to a law and can be estimated qualitatively and quantitatively. Such a preparation I term a chromatogram and the corresponding method, the chromatographic method". (200). In modern literature this method is called Tswett's adsorption analysis. The word "chromatographic" is ommitted, as various non-colored compounds have now been separated.

However, adsorption is just one of the phenomena that may occur in a Tswett column. In all there are three types of adsorbents (170). These are:

(a) Surface Active - those characterized by Tswett's adsorption analysis. Separation here depends on the variation in their adsorption capacities and in their selectivity or specificity for certain groups in the compounds being analyzed.

(b) Ion Exchange - those in which the particles exist as ions. Separation depends on the great combining capacity as well as on the rapid exchange rate and high reversibility of reaction with the solutes. The outer ions of ion exchange resin particles are not bound as tightly as the larger inner ones to the lattice frame-

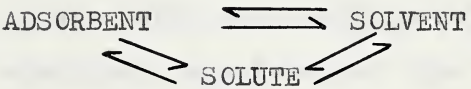
work and so they permit this rapid exchange with solute particles (96).

(c) Partition Adsorbents - those in which the adsorbent plays no part in separation, serving only as a mechanical support for the stationary liquid phase (112). Here the separation depends on the partition coefficient of the solutes in the chosen miscible or immiscible solvent pair.

The solvents used may partially alter the adsorbents so that more than one of the above types may be acting at one time. This partially explains why chromatographic separations in columns are still largely empirical. Modification in method of solvent application results in different developments of the chromatogram. Thus we may have frontal analysis, elution development or displacement chromatography (see glossary). The liquid chromatogram as developed by Tiselius and co-workers (40) shows how analysis can be accomplished by the correct choice of a solvent. As the solvent percolates through the column, it replaces the adsorbed solute particles so that the latter now appear in the eluant. The above examples show the different types of adsorption chromatography.

A comprehensive report on the various types of chromatography, with their chief underlying principles, is given by Meinhard (116). He mentions that the scope of modern chromatography can best be gained by considering this process from the standpoint of competing equilibria. In

general, the stationary phase (adsorbent) and the mobile fluid (solvent) compete with each other for solutes originally present in the fluid phase. The interactions are reversible and may be represented by the following relationship:



He states that the molecules or particles in the stationary and mobile phase may be one of four types, namely, ion, dispersion, complex, and association. Paper partition chromatography is included here but this will be discussed more fully in the next section. In his table showing the sixteen possibilities, all but five have been reported. Ionic reactions as ion exchange, inorganic chromatography, fractionation methods in which the solid phase is a dispersion, and diffusion of colloids in which both the solid and mobile phases are dispersions, as well as certain non-ionic reactions of a catalytic, partition, or salting out nature have been described. Leyon's (103) separation of a mouse virus, by using as solvent a concentration of ammonium sulphate that is just insufficient to precipitate the virus, is an example of the latter. As the solvent progresses up the filter paper carrying the virus with it, a point is reached where the concentration of ammonium sulphate is sufficient for precipitation because a certain amount of water had been adsorbed and held by the cellulose.

Ionophoresis has been used to separate basic amino acids from neutral and acid ones (42). The process consists of passing an electric current through a trough that contains silica jelly and the analyzing solution. The latter is placed in a gutter cut in the jelly at a considerable distance from the anode. The electrodes are inserted into buffers to prevent electrolysis and pH changes and are placed at the ends of the trough. As the current flows through the jelly, the amino acids and peptides move at different rates and to different poles. The current is stopped after some time and a strip of filter paper is put over the moist jelly and so one gets an imprint, when the paper is sprayed with the coloring reagent for amino acids (ninhydrin), of three distinct bands. The jelly can now be cut into sections and an analysis of these made by paper chromatography. It was found that the basic amino acids moved to the cathode and the dicarboxylic ones to the anode, whereas the neutral amino acids occupied the middle band. Similar electrolytic separation has been carried out on strips of filter paper (107).

Bioautographs (190). Development here is very similar to that carried out in ordinary papryography (53) or partography (146). However, no spraying reagents are used to bring out the color of the compounds analyzed. Instead, the paper is air dried, cut in pieces and placed in petri dishes which contain nutrient agar and a seeded test bacterium. The technique for growth factors, vitamins, etc., is very similar

to microbiological determinations. If antibiotics are separated, halos will appear on the agar. The paper strips can also be placed in test tubes containing a bacterium and special nutrient solution. Here turbidity or acidity titrations are taken as a criterion of growth or inhibition.

Radioautographs (8, 59, 60). These are special kinds of chromatograms in which the substances that are analyzed have their C, H, O or N labelled, i.e., in the radioactive form. On photographing these chromatograms on an X-ray sensitive film, dark areas appear wherever a compound contains the radioactive element. The paper chromatogram can now be sprayed with various coloring reagents depending on the group of compounds studied. An authentic radioactive derivative of the specific substance studied is developed simultaneously. The presence of one spot on the X-ray film in this new chromatogram is indicative of identity.

THEORY OF PAPER "PARTITION" CHROMATOGRAPHY

Consden, Gordon and Martin (41) basing their ideas on earlier work by Martin and Synge (112), consider that the separation of a mixture of solutes depends on their different partition coefficients between the mobile phase and the water saturated cellulose. Furthermore, they stress that adsorption of the solutes by the cellulose plays no important part in this separation. It is for this reason that they argue that the most satisfactory solvents (for amino acids)

are those that are partially miscible with water. Partition coefficient of a solute varies directly as its concentration in the stationary or water phase and indirectly as the concentration in the mobile phase. Therefore, the addition of an hydroxyl or a carboxyl group to a solute molecule increases its polarity and consequently its solubility in the water phase, while addition of a methyl group does the reverse. This is supporting evidence that the cellulose plays no part in the separation serving only as an inert mechanical support. Thus amino acid members of an homologous series will behave in a predicted way, e.g., alanine travels farther than its hydroxyl derivative serine (136). For miscible solvents they retain their partition theory by considering the process as a "salting out" effect. Bentley and Whitehead (13) argue that this explanation is too simple. They tend to uphold Hanes and Isherwood's concept (76), who consider that the water is bound in the filter paper possibly by hydrogen bonding to the hydrophilic hydroxyl groups of the cellulose forming a cellulose-water complex. The result is that the solute molecules with their hydrophilic groups will compete with water and solvent molecules (both of these are considered to have hydrophilic groups), for incorporation in this cellulose-water complex. Thus, as the water content of this cellulose complex decreases, its affinity for water increases. Therefore, the separation does not depend on whether the solvents are miscible with water, but rather on their affinities

for water. They stress (76) that the whole mechanism of chromatography centers on how this water cellulose complex holds solute molecules in competition with the flowing solvent. Therefore, the number and position of hydrophilic groups of both solvent and solute, as well as the water content will determine the R_f values. It should be stressed that this hypothesis does not incorporate either the partition or Tswett's adsorption theories.

Rutter (148) mentions that flow of one liquid over another may result in streaming potentials of varying magnitudes which may have significance as a possible mechanism of partition separations. He verified this by using water and inorganic salt solutions showing that this potential had a marked effect on separation of a dye mixture. Meinhard (116) considers paper partition as an example of an association - association interaction, i.e., where both the mobile and stationary phases are associations. He defines an association as "a mutual attraction between a solute particle and another substance, or between two solute particles of the same species, which does not involve the establishment of fixed bonds". It is characterized as a loosely bound complex of indeterminate composition. He suggests that such factors as hydrogen bonding, dipole or induced dipole interactions, or resonating attractions may be responsible for these low bond energies as contrasted with the greater bond energies involved in adsorption (ion exchange).

The fact that the movement (R_f values) corresponds very closely to the partition coefficients and can be predicted from the latter, is convincing evidence that the cellulose is inert. However, it is known that the water held in the cellulose is different from free water (76). The theories are therefore conflicting and it may be that more than one physical phenomenon is responsible. This would therefore account for the variable results sometimes encountered.

NOTE: A mathematical interpretation of paper chromatography based on the partition theory of Martin and Synge is given by Martin (111).

PAPER CHROMATOGRAPHY OF AMINO ACIDS

Amino acids were the first group of substances that the pioneers (41) separated by paper chromatography. Their method has been adapted for amino acid analysis of plant extracts. However, before a direct study of this kind could be undertaken, it was necessary to become acquainted with the techniques of paper chromatography.

Following a brief discussion of the method used in spotting, developing and spraying for amino acid analysis, a more detailed account of the solvents and coloring reagents tried, as well as factors affecting R_f values and some quantitative studies, will be described. All these

experiments were carried out on synthetic amino acids secured from Nutritional Biochemical Corporation, except cysteine hydrochloride and asparagine, which were obtained from Eastman Kodak and Merck respectively.

A pencil line is made about 9 cm. from one end of a strip of filter paper and just beyond this line a small circle of 1 cm. diameter is drawn. The amino acid mixture is then applied to this circle with a micropipette, the end of which is drawn out to a fine capillary and bent at right angles to facilitate spotting. As the solution is applied it is prevented from spreading beyond the limits of the circle by rapid evaporation which is accomplished by directing a stream of heated air to the spot, Figure 1, Approximately five microlitres can be spotted each time the pipette touches the paper and when this dries another amount is added. Amounts of 5 to 20 micrograms of each amino acid are sufficient for detection in paper chromatography. The spotted strip is now placed in the trough in the apparatus used for developing and is held there as shown in Figure 2. Both glass cylinders and large rectangular boxes were used. The solvent is added to the trough after the filter paper has become saturated with respect to solvent and water vapor (about 1 hour). When the solvent has progressed to the lower end of the filter paper, the latter is removed and the position of the solvent front is marked. The solvent is removed from the filter paper (the chromatogram) by sus-

pending it overnight in a large box heated by light bulbs to a temperature of 40 - 50° C. The dried chromatogram is sprayed with a 0.2% butanol solution of ninhydrin (triketo-hydrindene hydrate) which couples with the amino acids to form colored products.

For two-dimensional analysis the procedure is very similar. At a distance of 9 cm. from both edges of a corner of a 22 x 18½ inch sheet of filter paper, a circle 1 cm. in diameter is made. The amino acid mixture is applied in the same way but the amount is doubled. One edge of the filter paper is folded and is inserted in a long trough held in a large rectangular developing chamber, Figures 3 and 5. A glass rod holds the sheet of filter paper in the trough as before. After equilibrium has been attained with respect to solvent and water vapors, the solvent is added to the trough. A hole in the lid with a glass tube leading directly to the trough makes possible this operation without disturbing the saturated atmosphere, Figure 4. The paper is dried in the usual manner and the bent edge of the chromatogram is trimmed with a cutting board. The paper is turned through 90 degrees and the process is repeated with another solvent in a different chamber. For such a sheet about 50 ml. of spraying reagent is required. Often the spraying is repeated to ensure that all the ninhydrin positive material has reacted.

The amino acids possess different partition coefficients between the pure solvent and the water phases in

CHROMATOGRAPHIC APPARATUS

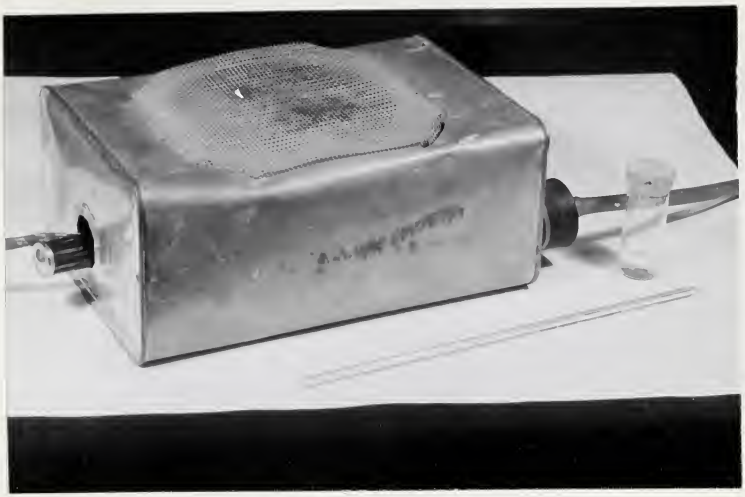


Figure 1. Spotting apparatus with hot plate and air inlet. A bent micropipette and a small vial containing a solute mixture are shown in the foreground.

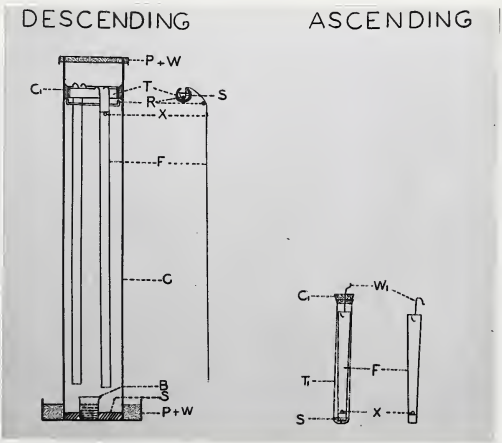


Figure 2. Chambers used for one dimensional chromatography. B, beaker with aqueous phase; C, glass cylinder; C_1, cork; F, filter paper; P-W, paraffin seals; R, glass rod; S, solvent or mobile phase; T, trough; W_1, wire; X, spot of solute.

CHROMATOGRAPHIC APPARATUS

Figure 3. External view of an air tight rectangular chamber used for two dimensional chromatography.

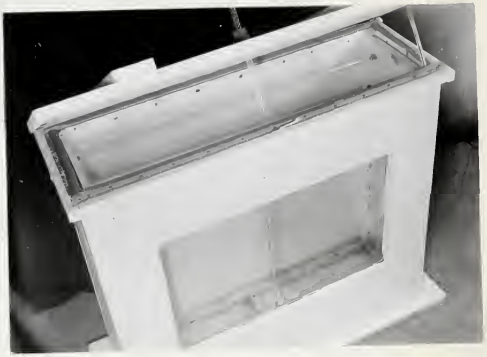


Figure 4. Chamber with raised lid showing delivery-tube leading to trough. Liquid containing the stationary and mobile phase are placed in separate containers in the bottom of the chamber.

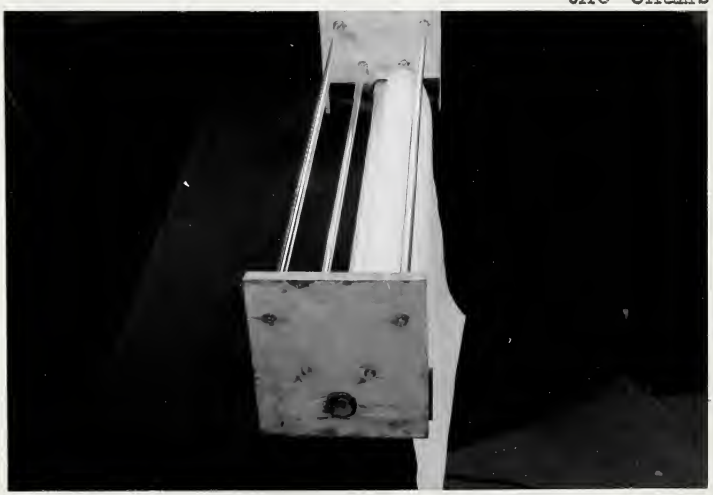


Figure 5. Trough showing a large sheet of filter paper held in place by glass rods. The apparatus provided for the simultaneous irrigation of two sheets.

the paper. As a result each acid will travel, "be adsorbed", to some definite point between the initial spot and the height reached by the solvent front. The ratio between the distance at which an amino acid is deposited and the total distance moved by the solvent is called the R_f value for that particular acid. This constant and the type of color which results from interaction with ninhydrin are the two main criteria of identification of the amino acids.

PREPARATION OF SOLVENTS

NOTE: The phenol crystals and n-butyl alcohol were products of Mallenckrodt.

Phenol (126)

Crystals of phenol were dissolved by placing the container in a warm-water bath. To the liquid phenol sufficient distilled water was added so that two layers were formed when the mixture was allowed to clear. The lower layer was used as the solvent and both the upper and lower layers were placed in separate containers in the bottom of the chamber to ensure saturated atmospheric conditions with respect to phenol and water vapor. Rubber gloves were used in all operations involving the handling of this chemical.

Phenol is a very good solvent to use as it gives a wide range of R_f values, Figure 6. However, the tailing

or "ghost" effects of certain basic amino acids, as well as appearance of faint spots in front of some regular amino acid spots may often be misleading. These irregularities can often be prevented by inclusion of a little ammonia in the aqueous portion of the solvent mixture in the bottom of the chamber. A few crystals of KCN were also added by Partridge to prevent oxidation (126).

Approximately 75 ml. of solvent are required for two large sheets and the time for this irrigation varies from 24 to 36 hours.

N-Butanol Acetic Acid Mixture (126)

The mixture is prepared by mixing n-butanol, glacial acetic acid, and water in the proportions of 4:1:5 by volume. The glacial acetic acid is added to the dry butanol and then the water is added. This is a three-component system and the amount of water contained in it is very important. The upper layer is the solvent mixture. As with the phenol solvent, both the upper and lower layers are placed in separate beakers in the bottom of the chamber to ensure saturated vapor conditions. The addition of acetic acid increases the polarity of the solvent and hence its water content is increased. The result is that higher R_f values are obtained than with ordinary butanol.

N-butanol acetic acid, abbreviated N.B.A., is one of the best uni-dimensional solvents that is known for the separation of amino acids, Figure 7. It was found to serve

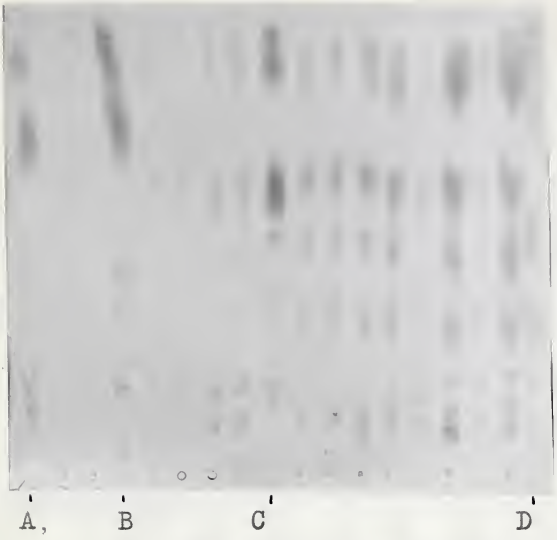
very well as a member of a solvent pair with phenol and as stated previously this combination was used exclusively as a solvent pair for two dimensional chromatography.

Butanol is very volatile and the evaporation of this solvent from the paper into the surrounding atmosphere inside the chamber during irrigation, results in a higher concentration of water than is required for saturation. The paper is said to be "water-logged", and even though the solvent progresses, no further separation of the amino acids occurs. Only by maintaining an air tight chamber can this essential butanol saturation be achieved. An assembly by which solvent may be added without removing the lid, similar to that described in the introduction of this section, is therefore a necessity with this solvent.

N-Butanol

This solvent is prepared by adding water to dry butanol until two distinct layers separate after shaking. The top layer is used as the solvent and again both top and bottom layers are placed in beakers at bottom of chamber to ensure saturation conditions. Preliminary work using the ascending method for determination of R_f values (145), showed that this solvent is not very useful for amino acid separation. (See Table 1 on R_f values). A more complete discussion of solvent-solute relationship is given on pages 32 to 35.

Of the above three solvents only N.B.A. must be used fresh for each run, because of the formation of an



Amino acids from lower to higher R_f region

Spot A- 3,13,23,22,12,20.

B- 24,21,7,14,22,10,9.

C- 19,26,6,2,17,8.

D- 1,6,5,16,11.

Figure 6. One dimensional chromatogram in phenol solvent. Spots A, B, C, and D contain known amino acids as shown above and the remaining spots are of wheat root extracts. Destruction and tailing of amino acids by this solvent is evident when compared with Figure 7.

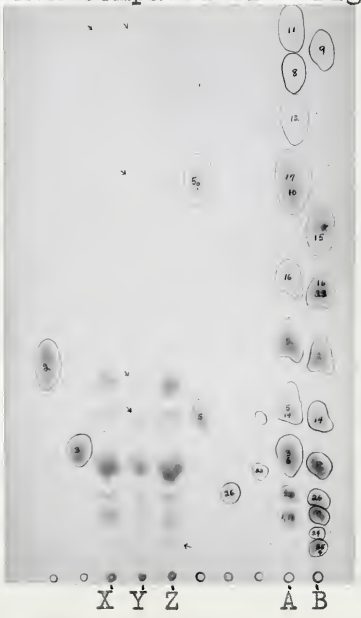


Figure 7. One dimensional chromatogram in N.B.A. solvent. Spots A and B contain mixtures of known amino acids while X, Y, and Z contain hydrolysed alcohol extracts of oats, healthy wheat, and diseased wheat roots respectively. The arrows indicate differences in amino acid content of these three samples. Key of the amino acids is given on p.44.

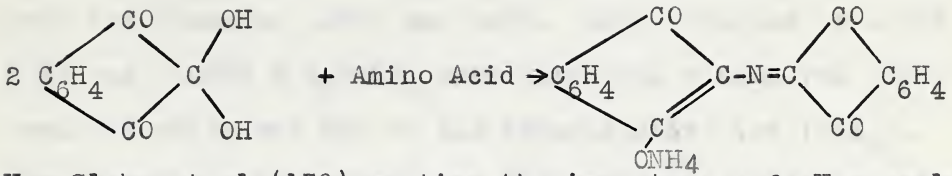
acetic acid ester on exposure to air. These three solvents have been used for separation of various groups of compounds, chief among these being sugars, antibiotics, and inorganic ions as illustrated in Table 2. Bryant and Overell have recently described a new solvent for amino acids (27).

PREPARATION AND DISCUSSION OF SPRAYING REAGENTS USED

Ninhydrin

The reaction of amino acids with ninhydrin was discovered by Ruhemann (179). An alcoholic solution of ninhydrin is usually prepared as this prevents spreading of the amino acid when the chromatogram is being sprayed and is also fast drying. A 0.2% solution was found very satisfactory, but the concentration is immaterial since the sensitivity is 1 in 100,000 parts (Gortner). This reagent must be stored in the dark since solutions that were kept in the greenhouse completely lost their yellow color as well as their ability to produce the characteristic Ruhemann's blue color. The production of this blue color is believed due to the coupling of two ninhydrin molecules through the alpha nitrogen of the amino acid. This reaction is specific for amino acids and for a very limited number of other organic compounds that possess in the free state a carboxyl and a neighboring amino group or the NH - CH_2 group. The color change that develops, therefore, is that of a substi-

tuted ammonium salt of diketohydrinedylidenediketohydriamine. The reaction according to Gortner (p 316) is:



Van Slyke et al (179) mention the importance of pH on color development and find that at low pHs, carbon dioxide is evolved. Various workers have stated that ammonium salts of organic compounds also give this ninhydrin test. The author was unable to produce the blue color with saturated aqueous solutions of ammonium oxalate, thiocyanate, and tartrate. Fiegl's spot test technique and spraying of a developed chromatogram were both negative.

Diazo Reagent

This reagent was prepared in two solutions as described in Hawk et al (78) and was used for the detection of amino acids containing indole groups (e.g. tryptophane and histidine). It was found that the reagent reacted very strongly with even traces of phenol solvent. Its use, however, was soon abandoned when it was found that with ninhydrin histidine gave a characteristic greenish "tinge" when one dimensional chromatograms were developed in N.B.A., and that tryptophane was well separated from the other amino acids in a two dimensional chromatogram and also gave a characteristic brown color.

For Sulphur - Containing Amino Acids

The iodoplatinate reagent as prepared by Winegard and Toennies (189) was used. Equal volumes of 0.066 M KI and 0.0033 M K_2PtCl_6 were mixed and a deep red color resulted which was due to the iodoplatinate ion (PtI_6). For spraying this reagent was diluted with distilled water 1:6. When phenol was used as solvent the paper was dried and then washed thoroughly with a one-to-one mixture of ether and acetone. This removes traces of the phenol solvent. After drying, the paper was sprayed with the above reagent and bleached areas against a pink background developed wherever sulphur-containing amino acids occurred. The above workers found that serine and threonine interfered. This reagent was used in this laboratory only for the determination of R_f values of cysteine, cystine, and methionine.

All these spraying reagents are typical biochemical tests used for the detection of amino acids. Although they were not tried the author sees no reason why specific tests as the aldehyde reaction for tryptophane (Hopkins-Cole) and Millon's for tyrosine, as well as many others (cf, Gortner), could not be utilized for confirming certain doubtful ninhydrin spots.

DETERMINATION OF R_f VALUES

Before irrigation was conducted with a three component system as N.B.A., the filter paper was completely

saturated with vapors of all the components of the solvent. Rapid evaporation of solvent from the paper during irrigation results in a decreased movement of solvent and a corresponding decrease in R_f values. After the strips have been developed, dried, and sprayed with ninhydrin and the blue color of the amino acids has developed to a maximum, these areas are encircled. A point is then marked in each circle which corresponds to the position of maximum solute concentration - the center of gravity (41). This location is very important since the partition process is almost entirely a partition phenomenon. If only surface active forces were responsible as with separation of inorganics in aqueous solvents, then the front of the spot would be used in R_f determinations. Correct location was found to reduce considerably the variation in R_f.

R_f values were determined using Rockland and Dunn's (145) modification of Williams and Kirby's (188) ascending technique, as well as the method of Consden et al (41) for descending technique. A comparison of R_f values obtained with those of other workers is shown in Table 1. Ten micrograms of each amino acid were used. The values obtained for the basic amino acids were much lower and in general lower values were obtained with all solvents in both ascending and descending irrigations. Various factors are responsible for variation in R_f and these will be discussed fully in the next sectio

TABLE 1

A Comparison of R_f Values of Amino Acids Obtained in This Study with Those by Other Investigators

Amino Acid	Phenol-Water						Butanol Acetic		Butanol Water
	Ascending			Descending			Ascending	Descending	Ascending
	a	b	c	a	d	e	a	a	a
Alanine		0.58	0.62		0.54 f	0.63			
Arginine	0.42	0.54	0.53	0.14	0.59	0.66	0.11	0.16	0.02
Aspartic Acid	0.18	0.22	0.25	0.10	0.15	0.19	0.12	0.14	0.03
Cysteine-HCl	0.53						0.29		0.14
Cystine	0.13				0.30		0.04		
Glutamic Acid	0.34	0.23	0.39	0.17	0.25	0.32	0.18	0.17	0.11
Glycine		0.36	0.49	0.16	0.40	0.42		0.15	
Histidine	0.38	0.62	0.81	0.16	0.69	0.77	0.07	0.05	
Iso-leucine	0.81	0.83	0.85	0.70	0.86	0.88	0.48	0.51	0.29
Leucine	0.77	0.80	0.86	0.70	0.85	0.88	0.49	0.52	0.33
Lysine	0.20	0.41	0.41	0.16	0.46	0.56	0.04		
Methionine	0.76	0.74	0.74	0.65	0.80	0.85	0.27	0.35	0.15
Nor-leucine	0.74			0.66	0.88	0.89	0.53	0.52	0.47
Phenylalanine	0.80	0.83	0.87	0.65	0.89	0.90	0.44	0.44	0.29
Proline		0.88	0.87		0.91	0.90			
Serine	0.32	0.30	0.33	0.13	0.36	0.37	0.11	0.11	0.04
Threonine	0.42	0.43	0.57		0.50	0.53	0.14	0.18	0.13
Tyrosine	0.55	0.55	0.53	0.53	0.64	0.63	0.31	0.38	0.22
Tryptophane	0.74	0.71	0.81	0.56	0.83	0.79	0.33	0.29	0.21
Valine	0.66	0.72	0.82	0.53	0.77	0.82	0.38	0.38	0.33

a - Hrushovetz
b - Williams and Kirby (188)
c - Rockland and Dunn (145)
d - Consden et al (41)
e - Pratt and Auclair (137a)
f - With KCN

Suffice it to mention here that Dent (53) found that variations up to 15% occurred in identical runs in the same chamber. He stressed the invalidity of the R_f as a criterion in analysis and mentioned that the relative positions of the amino acids remain constant in a one or two dimensional study (R_x values). It was this latter relation that was used as the chief criterion for determination of amino acids in wheat roots.

FACTORS AFFECTING R_f VALUES

Effect of Time

The normal procedure of studying the effects of time would be to remove the irrigating strips from the chamber at definite time intervals. Such a procedure, however, would disturb the saturated vapor conditions of the chamber. It was found that ammonium thiocyanate produced a pink color after it was spotted on filter paper, and that this color remained visible during irrigation with N.B.A. A dynamic relation between R_f and time was thus feasible.

The ascending technique of Rockland and Dunn (145) was used, as shown in Figure 2. Two ml. of N.B.A. solvent were placed in an eight-inch test tube. It was stoppered with a cork through which a wire was inserted. On this wire a strip of filter paper containing the spotted ammonium thiocyanate was suspended to reach the solvent but not to touch the walls of the test tube. The spot was two cm. from the

lower end which was immersed in the solvent, Figure 2. Measurements were taken at hourly intervals with the fourth reading taken at the half hour as the solvent front was already near the top of the filter paper. The results are shown graphically in Figure 8.

From this experiment it appeared that R_f decreased with time. Figure 9 shows that the movement of solvent and solute also decreased considerably during the irrigation process. An average of three trials was taken for these determinations. This decrease suggested that gravitational forces may be partially responsible. Some workers (157) have found that the solvent movement in an ascending system was half that obtained for descending irrigation. Such an increased time often has a deleterious effect on the solute mixture and it was for this reason that ascending methods were abandoned in analytical work.

Comparison of Ascending and Descending Methods

The comparison was made using the test tube method for ascending, and a large rectangular chamber for descending, chromatography. Ten strips for each amino acid were used in the test tube study whereas only five trials were tried in the descending method. The results, along with those of other investigators are shown in Table 1. With some amino acids as tyrosine, valine, methionine, and the leucines, the results are well within the 10% range of variability. However, the dicarboxylic and di-basic amino acids showed considerable

variation. Comparison of R_f values obtained by other workers showed similar differences. Thus Rockland and Dunn obtained a value of 0.81 for histidine whereas Consden et al obtained 0.69. It should be emphasized that in this test tube method it was impossible to obtain an atmosphere saturated with vapors of stationary and mobile phases. However, the importance of this factor was considerably reduced by using such small containers. It may be concluded that although there were great differences shown in R_f values obtained by the two methods, the relative positions occupied by the individual amino acids were the same in both tests.

Addition of Polar Solvents

Mathematical derivations by Consden et al (41) have shown that the following relation exists between R_f and partition coefficient (α).

$$\alpha = \frac{A_L}{A_S} \left(\frac{1}{R_f} - 1 \right)$$

But since $\frac{A_L}{A_S}$ is the ratio of volume of solvent and water phase in the moist chromatogram and is a constant for a given filter paper, it follows that:

$$\alpha = \frac{1}{R_f} - 1$$

i.e., there is an indirect relation. Thus, if the solubility of the amino acid in the solvent or mobile phase is increased, as by using a more polar solvent, the partition coefficient is

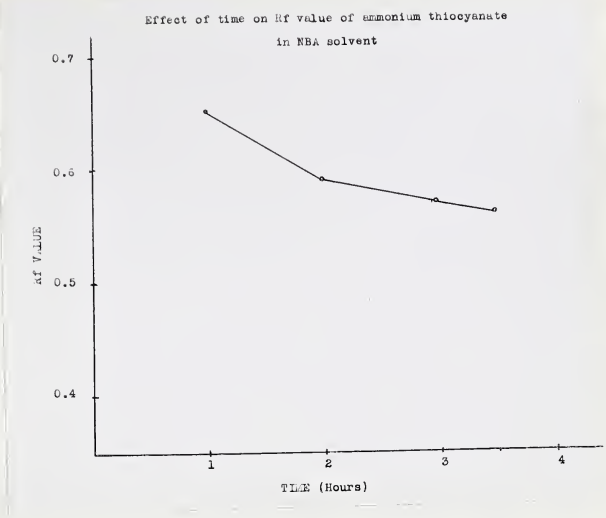


Figure 8.Effect of time on the R_f value of ammonium thiocyanate in N.B.A. solvent.

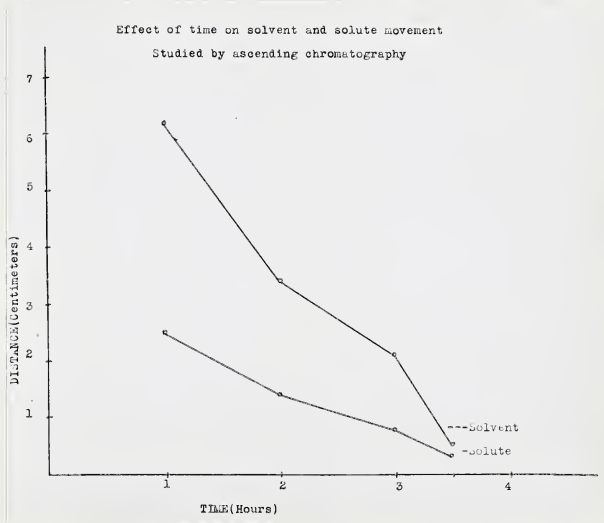


Figure 9.Effect of time on the movement of the solute ammonium thiocyanate and the solvent N.B.A.

decreased and the result will be an increase in the R_f value. To prove this, various concentrations of acetic acid ranging from 5 - 25% by volume, were added to butanol water. The following amino acids which represented different groups were tried:

Alanine - straight chain mono-amino-mono-carboxylic
Lysine - straight chain di-amino-mono-carboxylic
Aspartic acid - straight chain mono-amino-di-carboxylic
Phenylalanine - cyclic side chain mono-amino-mono-carboxylic
Tyrosine - hydroxy cyclic side chain mono-amino-mono-carboxylic

Whatman No. 4 filter paper was used in sheets. Amino acids were spotted nine centimeters from one edge and two centimeters apart. Twenty five spots were spotted on each sheet having five replicates of each amino acid. One hour was allowed for equilibrating the chamber and then 50 ml. of solvent were added to the trough without removing the lid. All sheets were removed when solvent had progressed to approximately five centimeters from the lower end. Results are shown graphically in Figure 10 and in the photograph, Figure 11.

The graph and photograph illustrate very strikingly that the addition of a polar solvent to the mobile fluid can greatly alter the R_f values. Values that were almost negligible in pure butanol (e.g. lysine), travelled almost half way up the paper when 25% by volume of acetic acid was added. The increased polarity of the solvent results in an increase

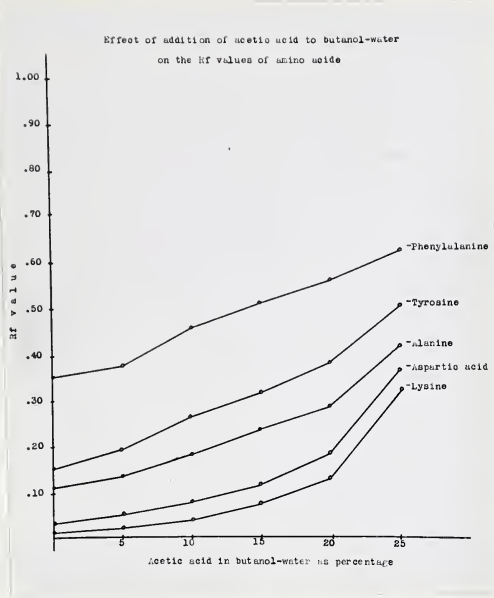


Figure 10. Graph showing the effect on the R_f values of amino acids of the addition of the polar solvent acetic acid to the organic solvent n-butanol.

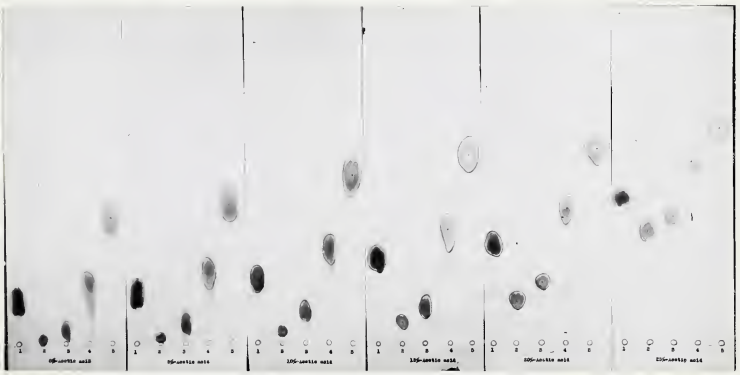


Figure 11. Chromatograms showing the effect of the addition of the polar solvent acetic acid to n-butanol on the R_f values of amino acids. Left to right: 0, 5, 10, 15, 20, and 25% acetic acid additions by volume.

in the amount of water it can hold, which in turn increases the solubility of the amino acid and hence its R_f value.

Concentration of Solute

With filter paper chromatography there is a definite limit of solute concentration above which no partition will occur. An attempt was made to find this limit for the amino acid alanine. The amino acid was spotted in various concentrations from five to 500 micrograms upon Whatman No. 4 filter paper. N.B.A. was used as the solvent. The results are shown graphically in Figure 12 and in the chromatogram, Figure 13. It was found that the R_f values increased with increasing concentrations of solute. The appearance of a ninhydrin-positive spot above alanine could not be explained. It was thought that some leucine impurity may have been present in the amino acid. Two ninhydrin-positive spots appeared below alanine and these too were thought to be impurities.

Distance of Spot from Trough

It was noted above (p. 30), that R_f values and rate of movement of solute and solvent decreased with time. A similar experiment was undertaken with amino acids to determine the effect on R_f values of placing the spots at various distances from the trough.

The amino acids, leucine and threonine, were spotted at varying distances from the edge of a sheet of filter paper as shown in Figure 15, so that the advancing solvent would

reach the solute at different times. These were spotted two cm. apart. After equilibrating the paper to the vapors of the chamber for one hour, N.B.A. solvent was added. The R_f values were then determined in the usual way. The graph and photograph show the results. (Figures 14 and 15 respectively).

It was found that a small decrease in R_f values occurred as the solute was spotted farther away from the trough. This may partly explain why the table of R_f values, p. 29, showed lower values than those of other investigators. Consden et al (41) spotted their solutes four to five cm. from the edge, as did most of the other workers. However, this effect is partially counterbalanced by the decrease in R_f value with the time of irrigation.

Type of Paper

According to Steward and Thompson (167) Kowkabany and Cassidy examined 75 types of paper in relation to amino acid chromatography and discussed the merits of each. In any paper chromatographic analysis only chemically treated paper should be used. When ordering these sheets (22" x 18½") of filter paper it should be mentioned that it is for chromatographic analysis. Various interfering substances as salts and metals may cause streaking of dicarboxylic amino acids, and furthermore, if the salts are present in high concentrations they tend to take water from the solvent and "waterlog" the paper so amino acids do not move. Williams and Kirby

(188) avoided this by equilibrating their solvents with saturated NaCl solution. Localization of the dissociated salts caused yellow colors with ninhydrin, whereas heavy metals caused ghost appearances which were due to the formation of soluble copper-amino acid complexes (41, 167).

Preliminary work showed that the rate of movement of solvent in Whatman No. 4 paper was greater than in the finer grade Whatman No. 1 filter paper. For this reason the R_f value of several amino acids was determined for these two types of filter paper. N.B.A. was the solvent. As in the previous section on polar solvent effects on R_f values, p. 32, a sheet of each type of filter paper containing five replicates of each amino acid was placed in the same trough and irrigated at the same time to reduce variables to a minimum.

It was found that in nine hours the solvent travelled 30 centimeters in Whatman No. 4 filter paper and only 23 centimeters in Whatman No. 1; see Figure 16. Figure 17 shows graphically the effects of the two papers on R_f values.

From this experiment it may be concluded that although the movement of solvent was greatly increased in Whatman No. 4 filter paper, the R_f value shows only a slight increase. Some amino acids as alanine and aspartic acid actually showed a decrease in R_f value in the thicker paper.

Compound Structure

According to Consden et al (41), the positions of the spots produced by various amino acids separated by paper

chromatography are determined by their partition coefficients between two phases, one of which is water saturated with organic solvent and the other organic solvent saturated with water. Addition of an OH or COOH group to the molecule increases its polarity and solubility in the water phase, while addition of a CH_2 group to the molecule decreases its polarity and thus its solubility in water. The former group causes a decrease in R_f values, the latter causes an increase.

In order to demonstrate this, a mixture of all the known free amino acids was spotted near one corner of a sheet of filter paper. The sheet was then subjected to two-dimensional chromatography using N.B.A. as first solvent and phenol-water (80% phenol) as the second solvent. The results obtained were similar to those of Polson (136), who used phenol with ammonia as first solvent and collidine as second solvent. The resulting chromatogram is shown in Figures 18, 19, and 19a.

In this chromatogram the dibasic acids, although not strict homologues, occupy the lower part of the sheet and fall on a straight line. Alanine occupies a position higher than its hydroxy derivative serine, as does also phenylalanine over tyrosine, and proline over hydroxyproline. Glycine and other members of the straight chain homologous series produce spots that fall on a smooth curve. The dicarboxylic amino acids occupy a position well to the left of the chromatogram.

The knowledge and understanding of the position

occupied by the individual amino acids greatly aids in suggesting the character and possible identification of unknown spots. It was in this way that the occurrence of gamma-amino-butyric acid in plants (89) and yeasts (139) was ascertained.

QUANTITATIVE PAPER CHROMATOGRAPHY OF AMINO ACIDS

Optical Density of Ninhydrin Color

Most of the quantitative techniques depend on a color produced with ninhydrin. Thus Polson compared the intensity of the spot with that produced by a known amount of amino acid (133), having abandoned the earlier technique of extracting the color with acetone and measuring the intensity colorimetrically (132). Others (6, 120) used first a dilute ninhydrin spray to locate the spots and then completed their reactions in test tubes. The procedure adopted by Awapara (6) consisted of locating the amino acids with 0.05% ninhydrin in butanol, cutting out the colored areas and placing them in test tubes to which two ml. of 1% ninhydrin solution and one ml. of 10% pyridine solution were added. The test tubes were then placed in a boiling water bath for 20 minutes after which they were transferred to a 25 ml. volumetric flask and made up to volume. They were read in a Beckman spectrophotometer at 570 mμ and the optical

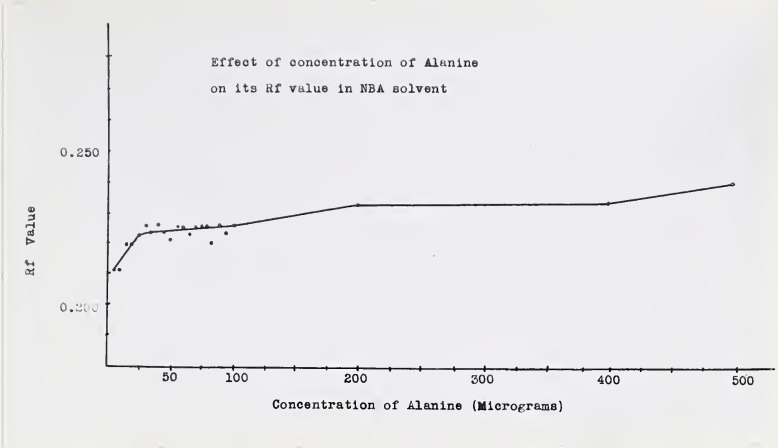


Figure 12. Graph showing the effect of the concentration of alanine on its R_f value in N.B.A. solvent.

Concentration of alanine(micrograms)

Figure 13. Chromatogram showing the effect of different concentrations of alanine on R_f value. The arrow indicates alanine spots. Other spots are caused by impurities or are ghost effects.

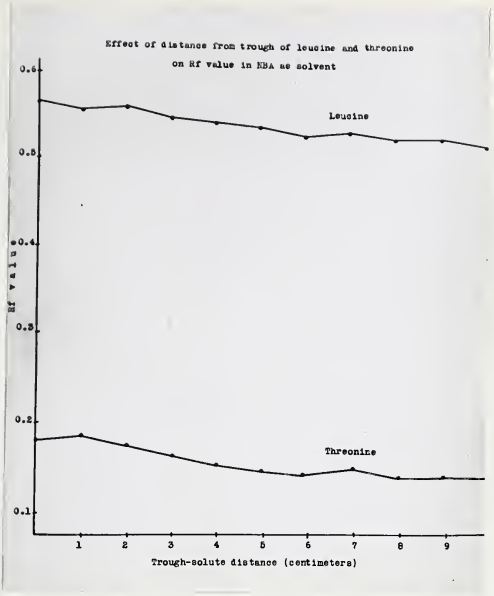
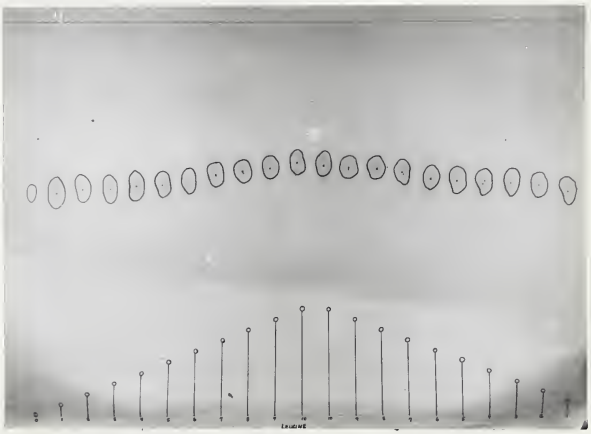


Figure 14. Graph showing the effect of spotting the solutes threonine and leucine at different distances from the trough on their R_f value.



Distance of leucine from the trough (centimeters)

Figure 15. Chromatogram showing the decrease in R_f value of leucine as this amino acid is spotted at greater distances from the trough. N.B.A. is the solvent.

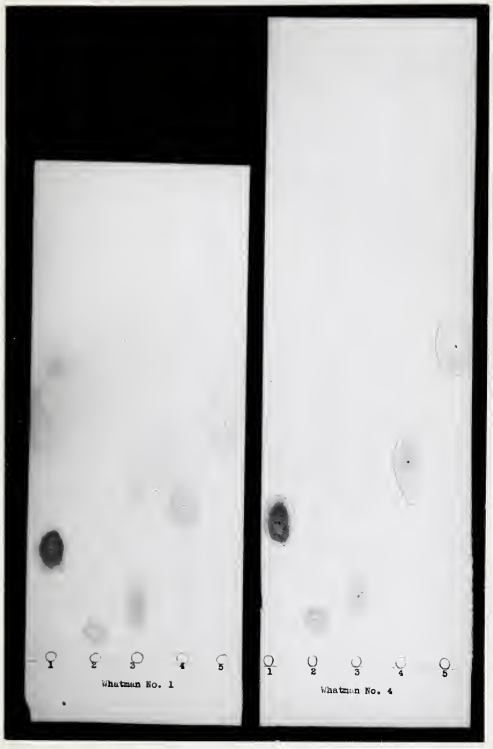


Figure16. Chromatograms showing the effect on R_f value of amino acids of different filter paper using N.B.A. as solvent.

1-alanine
2-lysine
3-aspartic acid
4-tyrosine
5-phenylalanine

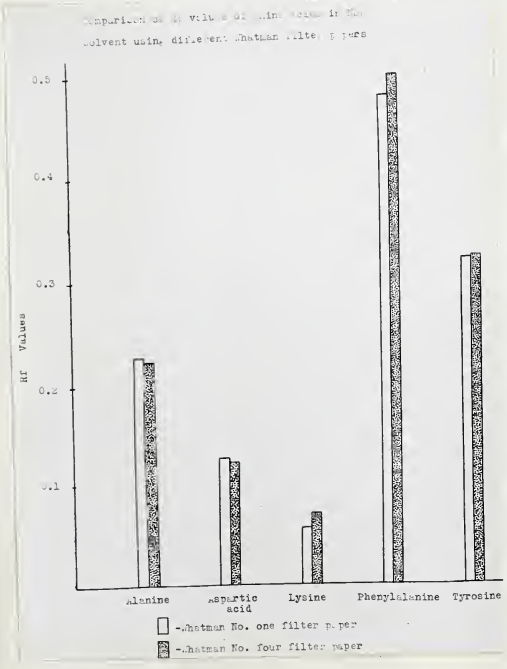


Figure 17. Graph showing the effect of different filter paper on R_f value of amino acids.

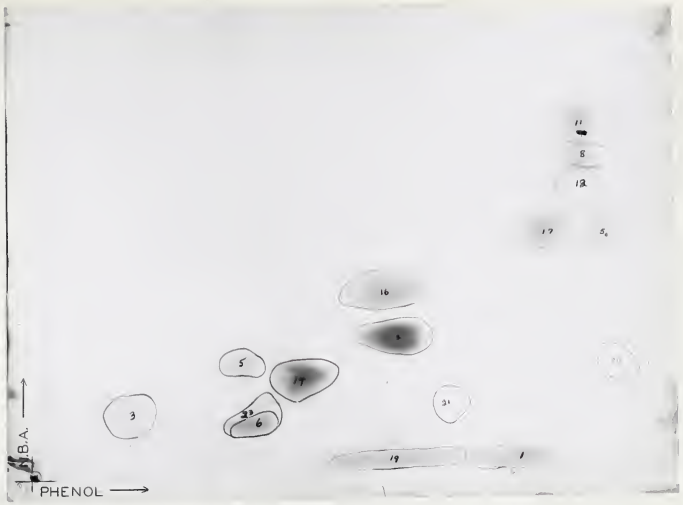


Figure 18.Two dimensional chromatogram in N.B.A. and phenol-water (80% phenol) solvents of 15 known amino acids.Key is found under Figure 19a.

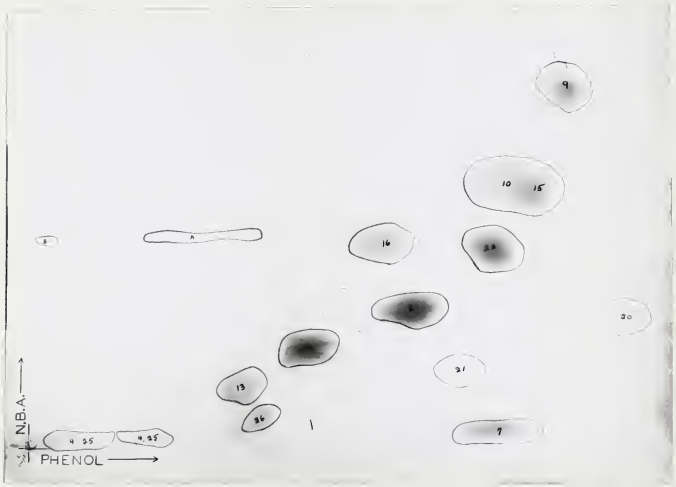


Figure 19.Two dimensional chromatogram in N.B.A. and phenol-water (80% phenol) solvents of 13 known amino acids. Key is found under Figure 19a.

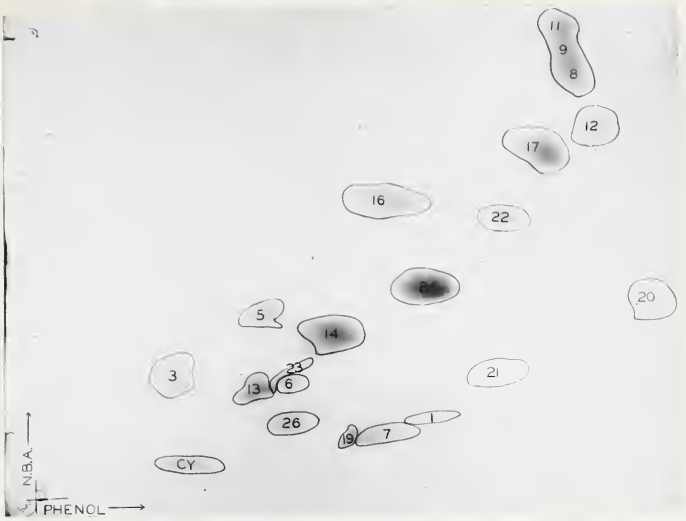


Figure 19a. Two dimensional chromatogram in N.B.A. and phenol-water (80% phenol) solvents of all the known free amino acids including asparagine and glutamine. Key reference is given below.

Key used for numbering of amino acid spots on chromatograms

1 -Arginine
2 -Alanine
3 -Asparatic acid
4 -Cysteine
5 -Glutamic acid
5o-Oxidized glutamic acid
6 -Glycine
7 -Histidine
8 -Iso-leucine
9 -Leucine
10-Methionine
11-Nor-leucine
12-Phenylalanine
13-Serine
14-Threonine
15-Tryptophane
16-Tyrosine
17-Valine
19-Lysine
20-Proline
21-Hydroxyproline
22-α-amino butyric acid
23-Glutamine
24-Glutathione
25-Cystine
26-Asparagine

CY-Cysteic acid

density was compared with a known standard. Preliminary work in this laboratory was done on color extractions using acetone and comparing light intensity with a standard in a Dubosq colorimeter. It was found that at least 50 micrograms of amino acid were required before any difference could be recorded. The Beckman spectrophotometer was not tried for this experiment.

Woiwood's Copper Phosphate Method

More detailed studies were done using Woiwood's copper phosphate method. His method (193,194) is similar to that of Pope and Stevens who allowed a suspension of $Cu_3(PO_4)_2$ to react with amino and "peptide amino" nitrogen. A soluble complex is formed at pH 9.3 between the copper and the alpha amino (or imino) group of the free amino acids or the free amino groups of peptides and proteins. This complex is filtered from the $Cu_3(PO_4)_2$ suspension and the amount of soluble copper is determined iodometrically. The copper so determined is related to amino or peptide N. Woiwood's modification consists of a colorimetric determination. The filtered complex is allowed to react with diethyldithiocarbamate and the yellow color that develops is measured colorimetrically. The copper so determined was then related to amino or peptide N, by means of a standard curve or a factor. He also found it necessary to replace the borate with Na_2HPO_4 as the former buffer made the copper reagent unstable. Standard curves must be prepared for each

amino acid as the ratio of weight of alpha amino N to weight of copper reacting, i.e., the F value or factor was not constant from acid to acid. Recently Spies and Chambers (161) found that excess of any amino acid added to a solution of this copper complex converts this copper largely to the complex of the added amino acid which then can serve as the standard. This therefore eliminates the preparation of standard curves for all the amino acids.

However, the modification which Woiwood applied to quantitative paper chromatography of amino acids was adopted. Volumes of standard copper solution containing one to 100 micrograms of copper were used and the curve relating concentration of copper to the "L" value is shown in Figure 20.

Numerically L = 2-log G, where G is the galvanometer reading. L is called the photometric density of the solution and is analogous to optical density as measured by the spectrophotometer. Tables are available in which the value of L corresponding to any value of G can be read at a glance. The standard curve showed an almost linear relation to 60 micrograms of copper. Further tests showed the sensitivity to be about one microgram, Figure 21. These values represent concentration of copper per 10^{7}/ml. With amino acids such sensitivity was not attained as it was found that amounts less than 40 micrograms of amino acids did not produce significant variation in colorimetric readings. Furthermore, the constant K value obtained with the copper curve and which is

characteristic of a linear relationship, was not obtained with either arginine, alanine or aspartic acid. The standard curve for these amino acids is shown in Figure 22. In the determinations of the standard amino acid curves, the amino acids were spotted on one inch squares of Whatman No. 1 filter paper. No attempt was made to obtain curves for developed chromatograms as it was thought that the destruction of the amino acids by the solvents would further decrease this sensitivity.

NOTE: In preparation of standard curves the following modification of aliquots from Woiwood's procedure was necessary as 15 ml. centrifuging tubes were used. For the standard copper curve the volume of the filtrate obtained after treatment with the $Cu_3(PO_4)_2$ suspension was reduced from two to one ml. and four instead of eight ml. of distilled water were added. For the amino acid standards two ml. of filtrate from the $Cu_3(PO_4)_2$ suspension were pipetted into two ml. of distilled water instead of the eight ml. which Woiwood used.

Other Quantitative Methods

Although Polson et al (132) were the first to report a quantitative procedure for amino acids employing the extraction of the ninhydrin color, they soon abandoned this for the "spot dilution" technique. This consisted of running a series of known concentrations of amino acids with the unknown

and then matching the intensity of the spots. Fischer et al (61) utilized the area of the spot. They showed that a linear relationship existed between the area of the spot of the test substance and the logarithm of the concentration at which it was applied. A theoretical derivation of this is given by Brimley (22). Rockland and Dunn (144) used a densitometric method, comparing density of an area larger than the biggest spot with that of the spot, while others used visual comparisons after adding known amounts of amino acids (168). Keston et al (94) used radioactive derivatives - the so called ultra microscopic dilution technique. Martin and Mittelman (113) used a polarographic method in place of the iodometric method of Pope and Stevens, when using the $Cu_3(PO_4)_2$ suspension procedure. The author sees no reason why Fitzpatrick's spectrophotometric quantitative studies of solutions of amino acids (62) could not be adapted for paper chromatography studies. It should be emphasized, however, that, none of these methods, with probably the exception of Polson's spot dilution technique, has been used successfully for two dimensional chromatography, although some workers have reported promising results with a combination of some of the above procedures (167).

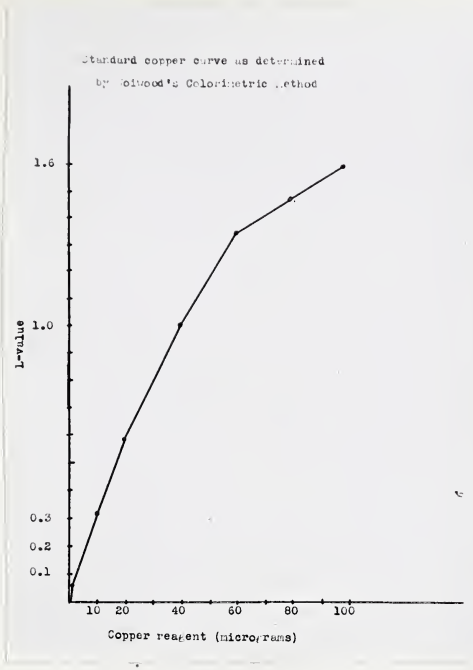


Figure 20. Standard copper curve prepared and determined by Woiwood's colorimetric method. Range is 0-100 micrograms of copper per 10 milliliters.

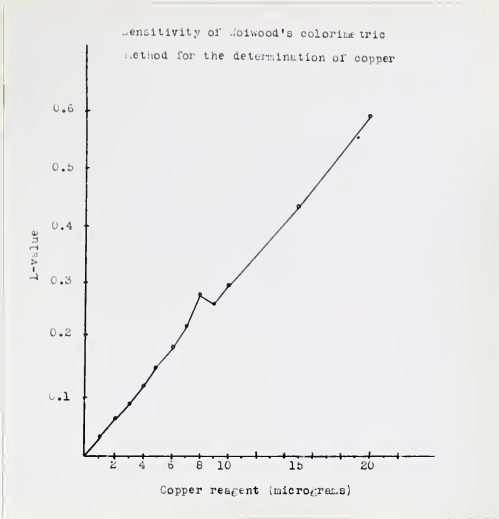


Figure 21. Standard copper curve prepared and determined by Woiwood's colorimetric method. Range is 0-20 micrograms and such was used to determine the sensitivity of this method.

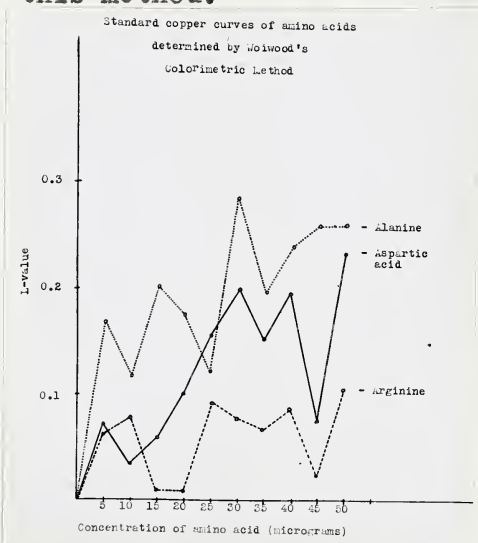


Figure 22. Standard copper curves for amino acids determined by a modification of Woiwood's colorimetric method for quantitative paper chromatography of amino acids. The absence of a linear relationship between concentration and the L-value, or the optical density, is evident.

Table 2

Substances separated by paper chromatography

and solvents employed

Substance	Source	Solvents employed
Amino acids	Alfafa(leaf)	As Consden et al(168)
Amino acids	Alfafa(root nodules)	As Consden et al(90)
Amino acids	Algae(Chlorella)	As Consden et al(56)
Amino acids	Algae	Phenol-NH_3 ,N.B.A.(65)
Amino acids	Animal connective tissue	As Consden et al(17)
Amino acids	Antibiotics (Gramicidin)	Benzyl alc.-HCN,s-collidine-3%NH_3,phenol(25) 43
Amino acids	Antibiotics (Circulin)	Glacial acetic-butanol-water(1:2:1) in powdered cellulose(129)
Amino acids	Bacteria(E.coli)	As Consden et al,also benzyl alc.,m-cresol(133)
Amino acids	Bacteria(E.coli)	Phenol-0.1%NH_3,collidine(95)
Amino acids	Bacteria(cultural filtrates)	Phenol,butanol-water-glacial acetic(125:125:30)(195)
Amino acids	Bacteria(hydrolysates)	M-cresol-0.1%NH_3,collidine(135)
Amino acids	Bacteria(hydrolysates)	As Consden et al(198)
Amino acids	Bacteria(metabolism)	N.B.A.,phenol(138)
Amino acids	Bacteria(Rhodotorula)	Pyridine-amyl alc.,m-cresol(122)
Amino acids	Bacteria(toxins)	N.B.A.,phenol(196)
Amino acids	Bacteria(transaminases)	Phenol(57)
Amino acids	Cancer tissue	Phenol,collidine-lutidine(142)
Amino acids	Chromosomes	Phenol(98)
Amino acids	Coenzymes	Phenol(39a)
Amino acids	Epidermis (mouse)	As Consden et al(143)

Substance	Source	Solvents employed
Amino acids	Nuclear sap (Amphibia)	N.B.A.,phenol(25)
Amino acids	Pepsin	Butanol-25%pyridine,butanol-20%acetic acid(91)
Amino acids	Pituitary)	Phenol,N.B.A.(102)
Amino acids	Apples	Phenol-NH_3,N.B.A.(89)
Amino acids	Plant tissue (meristematic)	Butanol-benzyl alc.(1:1)(1)
Amino acids	Pollen	As Consden et al(4)
Amino acids	Protein(hydrolysates)	Phenol,collidine,butanol, N.B.A.,(130)
Amino acids	Protein(albumin, globulin)	N-butanoln-propanol-0.1% HCl(1:2:1)(162)
Amino acids	Rat tissue	Redistilled phenol(6)
Amino,acids	Diseased rat and human tissue	Phenol,2-4lutidine(7)
Amino acids	Royal jelly	As Consden et al(137)
Amino acids	Rye grass	Phenol,N.B.A.(172)
Amino acids	Silk	M-cresol-0.1%NH_3,collidine(135)
Amino acids	Soil organic matter	Tertiary amyl alc.,collidine phenol(50)
Amino acids	Thyroid and plasma	N-butanol-2N formic,butanol-dioxan(4:1)(74)
Amino acids	Toxins-see bacteria	
Amino acids	Urine	As Consden et al(48)
Amino acids	Urine(normal and pathological)	As Consden et al(51)
Amino acids	Virus(bacteriophage)	Phenol-0.3%NH_3,collidine(134)
Amino acids	Virus(infected tuber)	Phenol saturated with salt solution(2)
Amino acids	Virus(tobacco mosaic)	N.B.A.,Phenol(165)
Amino acids	Virus(T.M.V. hydrolysate)	As Consden et al(83)

Substance	Source	Solvents employed
Amino acids	Wool hydrolysate	S-collidine, phenol in coal gas or NH_3(44)
Amino acids	Yeast	As Consden et al(104)
Amino acids	Yeast extract	Phenol, 2-6-lutidine, N.B.A.(139)
Alkali salts	Commercial	Butanol-NH_3, N.B.A.(97)
Alkaloids	Ergot	N-butanol(33)
Alkaloids	Precursors (Bellodoma)	Phenol(92)
Antibiotics	Penicillins	Amyl acetate-25% phosphate buffered at pH5.0(191)
Carboxylic acids	Plants	Phenol, butanol-propionic acid-water(153)
Carboxylic acids	Commercial(non volatile)	Ethanol-15N NH_4OH-water (90:5:5)(26)
Choline	Animal	N-butanol-morpholine(3:1), N-butanol-dioxane(4:1), N-butanol-pyridine(4:1), Phenol-all saturated with water(36)
DDT derivatives	Commercial	Ethanol-water-NH_3(80:50:5)(192)
Dyes	Commercial	Water on treated paper(147)
Ergothionine	Urine	Butanol(157)
Fatty acids	Silage	Butanol-water-ethylamine(181)
Inorganics	Commercial	Ionography(107)
Inorganics	Commercial	Organic solvents(3)
Inorganics	Commercial	Water soluble solvents(99)
Inorganics	Commercial	Organic and inorganic(101)
Inorganics	Commercial	Various organic and inorganic solvents(28)
Keto acids	As DNP derivatives	Butanol in 0.1M phosphate buffer(35)
Ketogluconics	Bacteria	Methanol and ethanol combinations(123)

Substance	Source	Solvents employed
Nucleic acids	Commercial	Butanol-NH_3, saturated butanol water-formic acid(90:10)(160)
Nucleotides	Flavine	N.B.A., collidine(47)
Organic acids	Plants	Butanol-acetic(106)
Pentose phosphates	Bacteria	Ethanol-acetic acid(80:0.8)(45)
Phenolic compounds	Bacteria	N.B.A., m-cresol-acetic acid-water(50:2:48)(11)
Phenolic compounds	Bacteria	Butanol-pyridine-saturated NaCl solution(1:1:2)(54)
Phenolic compounds	Tea catechins	N.B.A.(18)
Phosphoric esters	Fermentation products	Variety of organic acids and alcohols(76)
Photosynthetic products	Plants	Phenol, butanol(8) Phenol, butanol-propionic(12)
Photosynthetic products	Radioactive (plants)	Butanol-acetic acid-water (74:19:50)(165)
Pigments	Acridines	Water or aqueous HCl(101)
Pigments	Anthocyanins (petals)	N.B.A.(18)
Pigments	Flavanoid (plants)	Chloroform, ethyl acetate, phenol, N.B.A.(186)
Pigments	Fungi (Aspergillus)	Not specified(140)
Pigments	Pterins(insects)	N.B.A. with NH_4OH(69)
Pigments	Xanthopterin (silkworm)	N.B.A.(82)
Polyhydric alcohols	Commercial	Butanol-ethanol-water(4:1:5), benzene-butanol-pyridine-water(1:5:3:3)(87)
Proteins	Commercial	Ethylene glycol, ethanol or acetone with acetate buffer(67)

Substance	Source	Solvents employed
Proteins	Commercial	Phosphate buffer pH 9(93)
Proteins	Amylase etc.	60% saturated $(NH_4)_2SO_4$ at pH 6.5(117)
Purines,etc.	Commercial	Butanol(85)
Purines,etc.	Commercial	Butanol saturated with 1.5N NH_4OH (88)
Purines,etc.	Commercial	Butanol saturated with water-glacial formic acid(90:10)(110)
Purines,etc.	Commercial (quantitative)	Butanol(86)
Purines,etc.	Nucleic acid hydrolysates	Quinoline-collidine(3:1) saturated with water(182)
Radioactive compounds	Iodine(rats)	As Consden et al(59)
Radioactive compounds	Carbon(Chlorella)	As Consden et al(60)
Radioactive compounds	See photosynthetic products	
Radioactive compounds	Thyroid hydrolysates	Collidine-lutidine(175)
Radioactive compounds	Sulfur(urine)	Liquified phenol(176)
Ribonucleotides	Commercial	Aqueous iso-butyric acid buffered with ammonium iso-butyrate(109)
Sugars	Bacterial	N.B.A.,ethyl acetate-acetic acid-water(3:1:3)(55)
Sugars	Commercial(R_f)	N.B.A.(128)
Sugars	Commercial(Quant.)	N.B.A.(63)
Sugars	Commercial	Phenol-NH_3,N.B.A.,collidine(187)
Sugars	Methylated	Phenol,s-collidine,butanol-ethanol-water(4:1:5)(14,24)
Sugars	Bacterial metabolism	N.B.A.(64)
Sugars	Commercial	Phenol,N.B.A.with malonic or propionic acid(16)

Substance	Source	Solvents employed
Sugars	Various extracts	Phenol-1%NH_3, s-collidine, N.B.A.(125)
Steroids	Adrenals	Benzene-formamide, toluene-propylene-glycol, both saturated with water(30)
Steroids	Commercial	Benzene, tetralin, using alumina impregnated paper(31)
Steroids	Cortical(urine)	As for Adrenals above(199)
Steroids	Estrogens	Toluene-petrol ether-ethanol-water(20:10:3:7)(80)
Steroids	Progesterone	Ethanol(80%)(77)
Urochromogen	Urine	Butanol(157)
Vanillin and syringaldehyde	Spruce and - hardwoods	Petroleum ether saturated with water(15)
Viruses	Mouse virus	0.2 saturated solution of $(NH_4)_2SO_4$ pH 7.2(103)
Viruses	Bioautographs	N.B.A.(105)
Viruses	Bacteriophage	Phenol-0.3% NH_3, collidine(134)
Vitamins	Riboflavine	N.B.A., butanol-pyridine-water(3:4:7)(75)
Vitamins	B_{12}	Benzyl alc., butanol, secondary butanol(197)

Unless specified otherwise all organic solvents employed were saturated with water. Proportions where specified were by volume. The solvent pair used by Consden et al consisted of phenol saturated with water in a atmosphere of NH_3 or coal gas and collidine with water. N.B.A. solvent consisted of n-butanol, acetic acid (glacial) and water in proportions of 4:1:5 by volume.

CONCLUSION AND SUMMARY

The history, scope, and methods of partition chromatography have been discussed and a glossary has also been included. The procedure used in the paper chromatography of amino acids has been outlined. The preparation of solvents, spotting and irrigation of chromatograms, use of spraying reagents as well as quantitative determinations of amino acids were described in considerable detail. A study of factors affecting the R_f value proved its invalidity as a criterion for identity of a compound. The relative positions of amino acid spots in a chromatogram as well as their characteristic colors with ninhydrin, were taken as the chief criteria of identification of amino acids. Development of a chromatogram containing all the known free amino acids with N.B.A. as the first solvent and phenol-water (80%) as the second solvent showed almost a complete separation. It was concluded that this solvent pair could be used with reasonable success for determination of amino acids in an unknown biological extract free of peptides or proteins. Finally the table of fields of application of paper chromatography with the solvents employed was included to show the wide scope of paper chromatography in recent years.

P A R T I I

APPLICATION OF PAPER CHROMATOGRAPHY TO THE STUDY OF HEALTHY AND DISEASED WHEAT ROOTS

P A R T I I

APPLICATION OF PAPER CHROMATOGRAPHY TO THE STUDY OF HEALTHY AND DISEASED WHEAT ROOTS

INTRODUCTION

Studies of amino acids of plant tissues using paper chromatography were not undertaken until 1947 when Dent et al (52) found 21 known amino acids and three unknown spots in a 70% alcoholic extract of potato tubers. They also observed that on hydrolysis no extra spots occurred (167). By the use of cold 70% alcohol they obtained a protein-free filtrate and found that approximately two thirds of the total nitrogen of the tubers was soluble in alcohol.

HISTORICAL REVIEW

The importance of various roots to the wheat plant has been studied by Sallans and coworkers (150). Their experiments involved the amputation of different roots as lateral, auxiliary, or seminal, and the study of such amputations on growth of the plant. However, it was

the application of the chromatographic technique that has helped in the understanding of nitrogen metabolism in the roots. The symbiotic relation of roots and nodules in leguminous plants was studied by Hunt (90) who found free tryptophane as well as certain basic amino acids in mature nodules but not in roots. Allsopp (1) found more amino acids in the root apices of certain ferns than in the general root portions.

However, Andreae (2) was one of the first workers to report a study of the amino acids of healthy and diseased plant tissue. In his chromatograms he found that healthy potato tubers had a predominance of tyrosine and tryptophane while the diseased ones showed no tryptophane and very little tyrosine. He suggested that the leaf roll virus which was responsible for this disease may have interfered with the synthesis of these amino acids. It was also suggested that the tyrosinase enzyme may be more active in the infected tissue. No references have been noted on studies of the amino acids in healthy and diseased wheat roots.

EXPERIMENTAL PROCEDURE

Obtaining Healthy and Diseased Wheat Roots

Several fungus pathogens of wheat roots are commonly found in Edmonton black loam, but for the purposes of this study one of these, Helminthosporium sativum (P.K. & B.), was

used. Inoculum of this pathogen was prepared in the following manner:

An isolate of H. sativum which was known to be parasitic to wheat roots was increased in quantity by subculturing in test tubes on Czapek's synthetic agar medium. These tubes were incubated for a period of 14 days at room temperature. Ten ml. of sterile distilled water were added to one of these test tube cultures and after the contents were mixed with a transferring needle, the resulting suspension was added aseptically to five flasks. Each flask contained 140 grams of a moist sterilized mixture of three parts soil and one of sand, plus 2% corn meal by weight. This was incubated for a period of 10 days at 65° F, during which time the fungus penetrated the soil and usually developed a fine grey mat of fungus mycelium and spores. This mixture is called soil inoculum of H. sativum. The above process of its preparation is shown diagrammatically in Figure 23.

One of these flasks was then transferred to a pot of virgin black soil. The inoculum was mixed well with this soil and then soaked with water. After a period of two days, which allows the fungus to establish itself in the soil, ten surface-sterilized wheat seeds were seeded in each pot. Surface sterilization of the seed was accomplished by soaking the seed overnight in water, draining, adding 0.01% $HgCl_2$ for 10 minutes, and washing well with sterile water. Thatcher wheat was used.

After a two to three week growth period the plants were removed and the first three inches of the root below the crowns were removed and washed. Roots that did not show any lesions or discolorations were discarded. Healthy roots were obtained by following the same procedure except that the soil inoculum of the fungus is replaced by a flask of the sterilized soil mixture.

PREPARATION OF WHEAT ROOT EXTRACTS FOR CHROMATOGRAPHIC ANALYSIS

Initial extraction work was carried out using large amounts of fresh root tissue and small volumes of extracting fluids. Four ml. of the extracting solvent were added to a five gram sample of fresh moist root tissue. This was ground to a fine paste in a mortar by the addition of a few grains of sand. This was then filtered through a Buchner and about 4.5 ml. of filtrate were obtained. Volumes of 50 microlitres were spotted on paper strips. Figure 24 shows the effects of hot and cold water and cold 80% ethanol, and from these results it appeared that hot water was the best for extraction. However, in these strip chromatograms it was observed that the water extraction method caused considerable spreading of the spots. Similar results were obtained when moist roots were ground in a mortar without any solvent and the extracted juice was chromatographed, Figure 25. This suggested that inorganic ions or other compounds might have interfered

with the partition (126).. The extract was therefore put through ion exchange colums using Partridge's method (126). Percolation of the extracted juice through a Zeo-karb column, which was used for removal of cations and bases, did not result in much loss of amino acids. However, after this was percolated through Deacidite column, which removes anions, it was found that the amino acids were almost completely lost as only a few faint spots were visible in the region of lower R_f values. This is shown in Figure 26.

It was thought that after running through the first column the amino acid existed in the cationic form so that percolation through the Deacidite column would cause an exchange of the sodium ion in this resin with the positively charged amino acid. Apart from this fault, future work utilizing the water extraction method proved unreliable as older preparations usually resulted in loss of amino acids.

The alcoholic extraction method of Dent et al (52) was tried and was found to be most satisfactory. It was slightly modified.

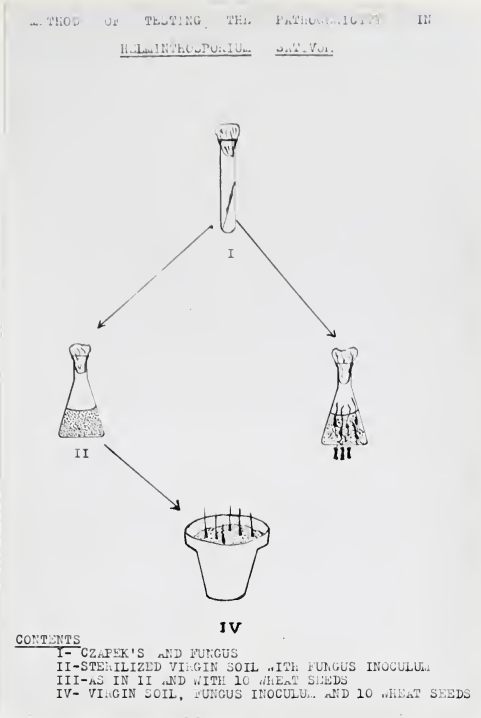


Figure 23. Diagram of the method used in testing the pathogenicity in Helminthosporium sativum.

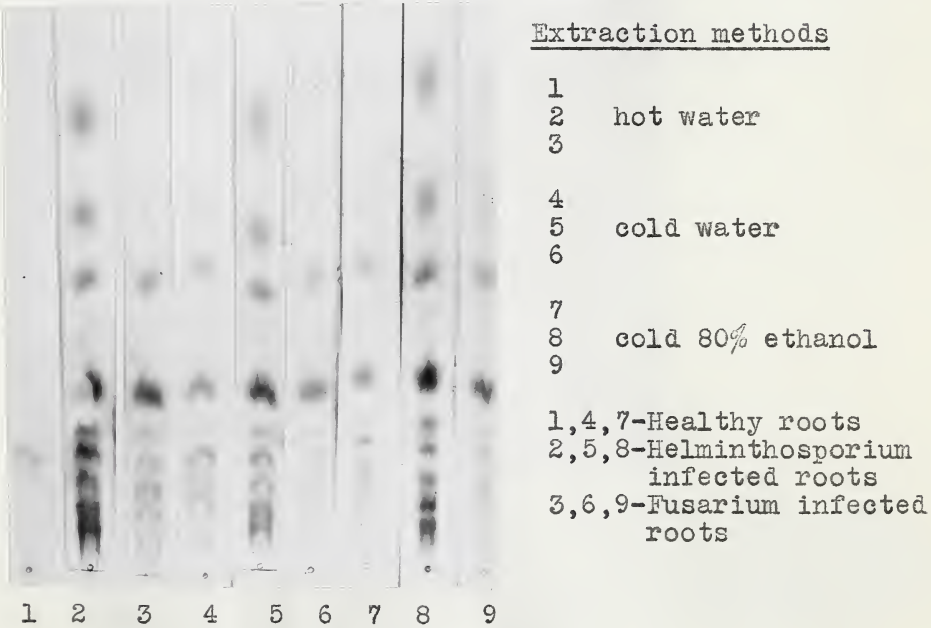


Figure 24. Chromatogram in N.B.A. solvent showing the efficiency of various extraction methods for amino acids in wheat roots.

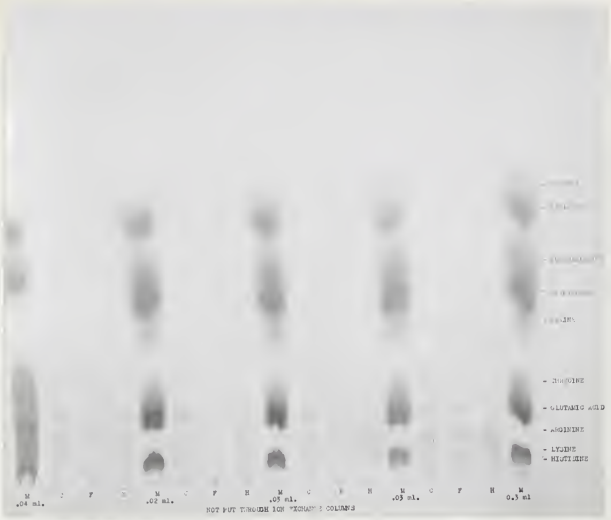


Figure 25. Chromatogram run in N.B.A. solvent and containing spots of known amino acids and also of various wheat root extracts. C, F, and H are spots of extracts prepared by squeezing fresh samples of healthy wheat roots, Fusarium infected roots, and Helminthosporium infected wheat roots respectively.

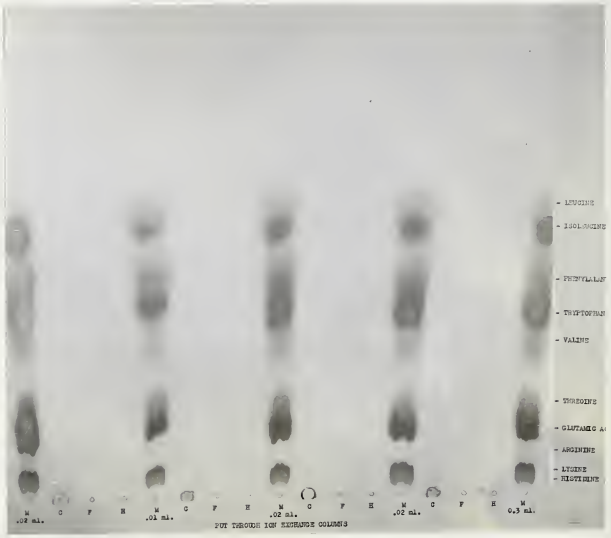


Figure 26. Chromatogram as above only the extracts were first washed through Zeo-karb and De-acidite ion exchange columns. The known amino acids which served as markers wwere not put through ion-exchange columns. The amino acids were completely removed by this treatment.

Extraction Method I - Large Volume Technique

To five grams of fresh roots 100 ml. of 80% ethanol were added and one ml. of concentrated NH_4OH to neutralize any acids that might cause hydrolysis of peptides or proteins. The resulting pH, using a Beckman pH meter, was 6.72 for the healthy and 6.65 for the diseased extracts. The flask containing this mixture was placed in a water bath, the temperature of which was just sufficient to make the alcohol boil. After 30 minutes of boiling the alcohol was decanted and filtered. Another 100 ml. of alcohol were added and this extraction process was repeated four times. The filtrates were pooled and reduced to a volume less than 100 ml. by reduced pressure and slight warming. The resulting solution was placed in a 100 ml. volumetric flask and made up to volume with 80% ethanol. The resulting pH was 6.25 for the extract of healthy tissue and 6.50 for the extract of diseased tissue. Chromatograms were prepared with 1000 microlitres of solution per spot and the results are shown in Figures 27 and 28.

Extraction Method II - Small Volume Techniques

A - Fresh Tissue

Fifty mgm. of fresh roots were placed in a small 15 ml. vial and two ml. of 80% ethanol were added. This was immediately placed in the refrigerator to freeze the tissue. Just before these samples were used for chromatographing, the roots were macerated in the vial with a stirring rod. The sus-

pension was allowed to settle and the clear fluid was carefully pipetted and spotted directly on filter paper. Chromatograms were spotted containing 1200 microlitres of solution and the results are shown in Figures 29 and 30 (frozen samples) and Figures 31 and 32 (not frozen samples).

B - Dry Tissue

Dry roots were obtained by placing fresh roots overnight in an oven heated at 90° C. They were partially ground with a mortar and pestle and to 10 mgm. of this powder two ml. of 80% ethanol was added. At least 24 hours were allowed for extraction. The vial was shaken periodically to hasten this process. After the suspension had settledthe top clear fluid was carefully removed with a micropipette and spotted directly on paper. Chromatograms containing 1000 microlitres of solution are shown in Figures 33, 34, and 41.

The value of each technique will be discussed in Section 2 on chromatographic analysis.

HYDROLYSIS

The acid, alkaline or enzyme methods are generally employed in protein hydrolysis. Stepka and Takahashi (166) discuss the merits of each using the tobacco mosaic virus protein. Only acid hydrolysis was employed in wheat root studies using macro and micro quantities.

Macro: Figures 35 and 36

Five gram samples of fresh root tissue were placed in 200 ml. flasks and to each 50 ml. of 6N HCl were added. These flasks were loosely stoppered with glass wool and the contents were hydrolyzed in the autoclave overnight at 12 pounds pressure. (Glass wool was used because ordinary absorbent cotton charred considerably.) The plugs were removed and the contents evaporated to dryness in a 90° C. oven. Then 100 ml. of 80% ethanol were added, and the contents stirred and allowed 24 hours for complete extraction. This was repeated with one gram samples and the volumes of acid and ethanol were reduced by half.

Micro: Figures 37 - 40

Amounts of 0.01 and 0.05 gms. of dry tissue were placed in small vials and two and five ml. of 6N HCl were added respectively. Hydrolysis was carried out as outlined above. Following evaporation, the contents were dissolved in two and five ml. of 80% ethanol respectively. A similar hydrolysis was carried out on 0.05 gms. of fresh tissue, using two ml. 6N HCl and dissolving in two ml. of 80% ethanol.

SECTION 2 - CHROMATOGRAPHIC ANALYSIS

Spotting Technique

With the macro extraction techniques it was found that a large volume of solution must be spotted before any positive results could be obtained. The following description

of an inexpensive yet efficient spotting apparatus for drying the solute is therefore included.

Figure 1 shows the general external appearance. An aperture four inches in diameter is made in one side of a rectangular gallon tin. Through this aperture a small hot plate is placed within the can and a hole is made in the bottom of the latter to allow the electric cord and the temperature regulator of the hot plate to protrude. A screen is then fitted over the side aperture and held in place by bending the corners into slits made in the tin. A rubber stopper is inserted in the vent of the tin. Through this is inserted a glass tube that is bent and drawn out to a fine capillary and an adjustment is made so that when a current of air is forced through it, and directed on the hot plate, the resulting warm air moves upward on the screen. With this apparatus 1500 microlitres of solution can be spotted in one hour.

One and Two Dimensional Studies

The large volume extraction technique, Figures 27 and 28 is not recommended as only a few spots show up even after 1000 microlitres of solution are spotted. Since five grams of fresh tissue were used and the final volume of the extract was 100 ml., the results would probably be the same if one ml. of extracting solution were used for 0.05 grams of fresh tissue. An experiment to test this was carried out and the resulting chromatograms are shown in Figures 31 and 32.

acid derivative.

To determine the bound amino acids, it was necessary to remove the free amino acids first. Two ml. of 80% ethanol were added to 0.01 grams of dried root tissue. After 24 hours the alcohol was removed and the residue was subjected to hydrolysis as described above. Chromatograms containing 600 microlitres of solution were spotted and are shown in Figures 39 and 40 (bound amino acid chromatograms). They should be compared with Figures 37 and 38 (free and bound amino acid chromatograms) which show both the free and bound amino acids and with Figures 33 and 34 (free amino acid chromatograms) which show only the free amino acids. These six figures were used for preparing Tables 3 and 4. In all cases 0.01 grams of dry tissue was used and the hydrolysis treatment of spotting, etc., of Figures 37 - 40 were similar. The chief difference shown is the decrease in basic amino acids as well as the absence of spot W from the "bound-amino acid" chromatograms. There was also a decrease in spots B and C and A was almost absent. These three spots could not be identified. Spot C was thought to be aspartic acid as it was found that this amino acid sometimes showed two spots (53). Threonine and alanine also showed decreases. The leucines, valine, and proline were unchanged. This is to be expected since these amino acids were found in low concentration in the free state. Proline, as well as tyrosine and tryptophane, could not be detected in the free state. Asparagine and alpha amino butyric acid were destroyed by acid

At least five more spots occurred than in the large volume extraction process. If tissue is frozen immediately after removal from the wheat plant, Figures 29 and 30, an increase in asparagine and the basic amino acids occurs. Similar increases were obtained when the roots were quickly dried and ground to a powder and the alcoholic extract was spotted, as is shown in Figures 33 and 34. Figure 41 is a chromatogram of oat root tissue treated by this drying process. Repeating chromatograms several times according to all the above mentioned procedures, showed that the latter method of drying and grinding the tissue to a powder previous to extraction with alcohol gave the most consistent results. Furthermore, this procedure does not destroy asparagine or glutamine and at the same time it increases the concentration of the basic amino acids.

For a study of the bound and the free amino acids, it was found that there was no advantage in using either the large or the small volume extraction procedures. See Figures 35, 36, 37 and 38. The greater concentration observed in Figures 37 and 38 occurred because a much larger volume was spotted. There is, however, one other difference between the chromatograms and that is the absence of spot W from the large volume extraction technique. The larger concentration of solute may be responsible for this. The position occupied by this substance does not correspond to any of the known free amino acids. Its position suggests it may be a basic amino

hydrolysis.

For quantitative determination of amino acids, the spot dilution technique of Polson et al (132) was used. The intensity of the ninhydrin spot was compared with several standards. For the concentrations less than 60 milligrams per gram of dry tissue the accuracy is ± 5 milligrams, whereas for values higher than this, the accuracy is much reduced as no standards were prepared for such high concentrations.

TABLE 3

Comparison of Unknown Ninhydrin Spots in Chromatograms of Healthy and Diseased Wheat Root Samples

	Healthy Roots			Diseased Roots		
Spots	Free	Free and Bound	Bound	Free	Free and Bound	Bound
A	-	+	+	-	+ +	+
B	-	++++	+	-	+++++	+
C	-	+++	+	-	++ + +	+
T	-	+ +	-	-	+	-
W	-	+	-	-	+ +	-
Y	+	-	-	+ +	-	-

TABLE 4

Concentration of Free and Bound Amino Acids in Healthy and Diseased Wheat Root Samples

	Healthy Roots			Diseased Roots		
Amino Acid	Free	Free and Bound	Bound	Free	Free and Bound	Bound
Alanine	2.0	6.0	6.0	4.0	13.0	13.0
A.A.	-	5.0	-	-	3.5	-
Arginine Histidine Lysine	+	+++++	+++	++	++++++	++
Asparagine	6.0	-	-	12.0	-	-
Aspartic Acid	3.0	6.0	5.0	5.0	8.5	6.0
Glutamic Acid	4.0	8.5	8.5	4.0	11.5	8.5
G	-	13.0	3.5	-	6.0	5.0
Glycine	-	4.0	4.0	-	6.0	5.0
Glutamine	8.0	6.0	6.0	8.0	6.0	6.0
Leucine	1.0	8.5	6.0	1.5	12.5	8.5
Phenylalanine	-	6.0	6.0	-	8.5	3.5
Proline	-	5.0	5.0	-	6.0	6.0
Serine	1.5	3.5	5.0	4.0	6.0	6.0
Threonine	2.0	8.5	3.5	2.5	12.5	5.0
Tyrosine	-	3.5	3.5	-	5.0	5.0
Valine	1.0	5.0	2.0	15	6.0	1.5
[a]Gamma-Amino Butyric Acid	2.0	-	-	15	-	-
TOTAL	35.5.	88.5	64.0	44.0	111.0	79.0

Concentration is expressed as milligrams per gram of dry tissue and is estimated by the spot dilution technique using paper chromatography (132).

a. Uncertain whether alpha- or gamma-amino butyric acid, as they have identical R_f values.

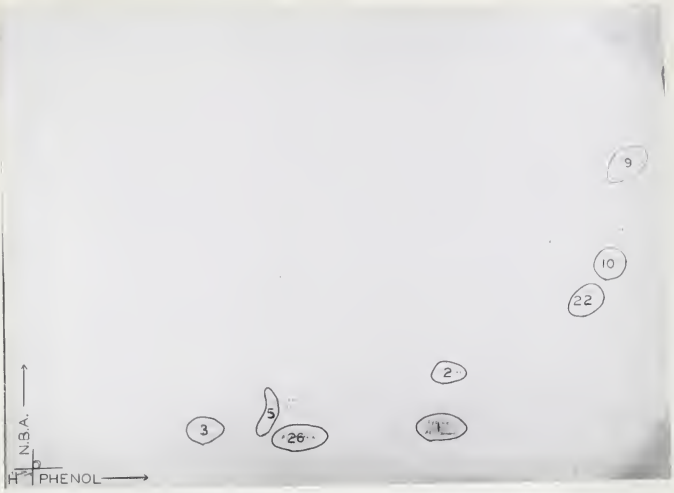


Figure 27. Chromatogram of 1000 microliters of healthy wheat root extract prepared by large volume extraction procedure. Solvents are N.B.A. and phenol-water.

Figure 28. Chromatogram of 1000 microliters of diseased wheat root extract prepared as in Figure 27.

Figure 29. Chromatogram of 800 microliters of healthy wheat root extract prepared by micro extraction methods of fresh root samples. Roots were frozen previous to extraction. Solvents are N.B.A. and phenol-water.

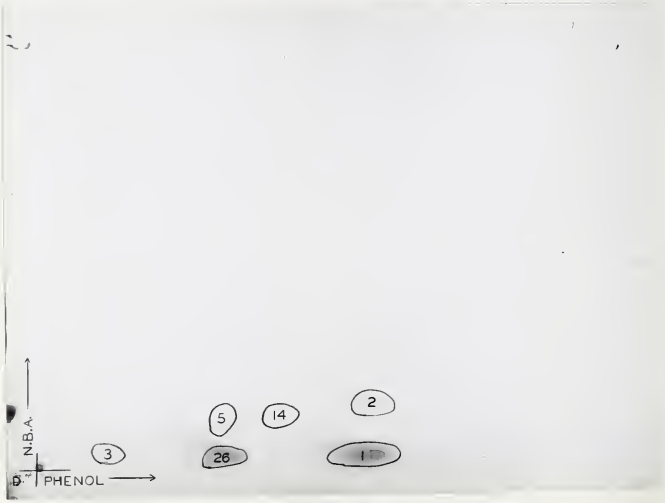


Figure 30. Chromatogram of 800 microliters of diseased wheat root extract prepared as in Figure 29.

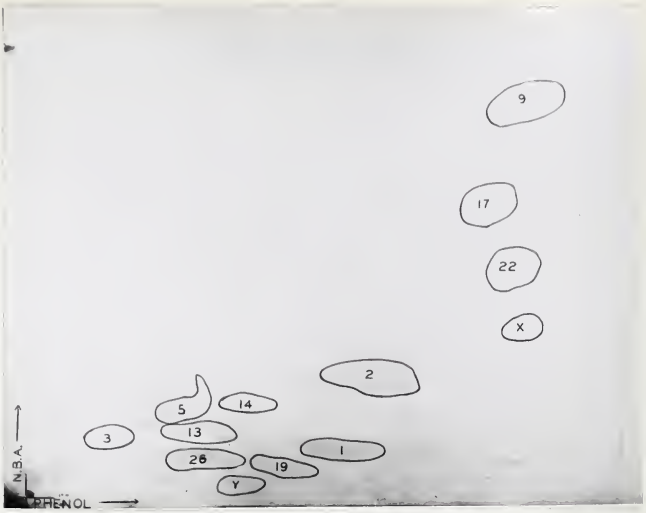


Figure 31. Chromatogram of 1200 microliters of healthy wheat root extract prepared by micro extraction methods of fresh root samples. Solvents are N.B.A. and phenol-water.

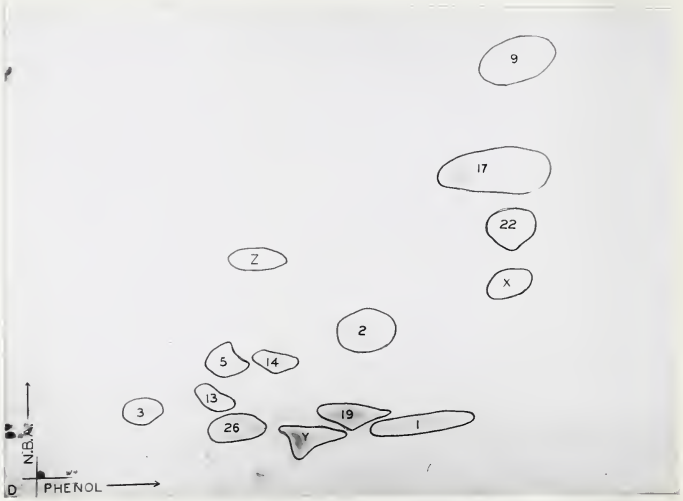


Figure 32. Chromatogram of 1200 microliters of diseased wheat root extract prepared as in Figure 31.

Figure 33. Chromatogram of 1000 microliters of healthy wheat root extract prepared by micro extraction methods of dried root samples. Solvents are N.B.A. and phenol-water.

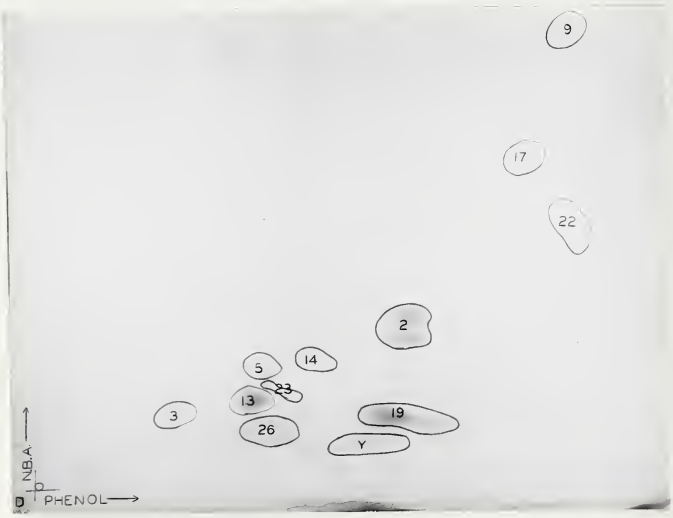


Figure 34. Chromatogram of 1000 microliters of diseased wheat root extract prepared as in Figure 33.

Figure 35. Chromatogram of 500 microliters of healthy wheat root extract prepared by macro hydrolysis method of a five gram sample of fresh root tissue. Solvents are N.B.A. and phenol-water.

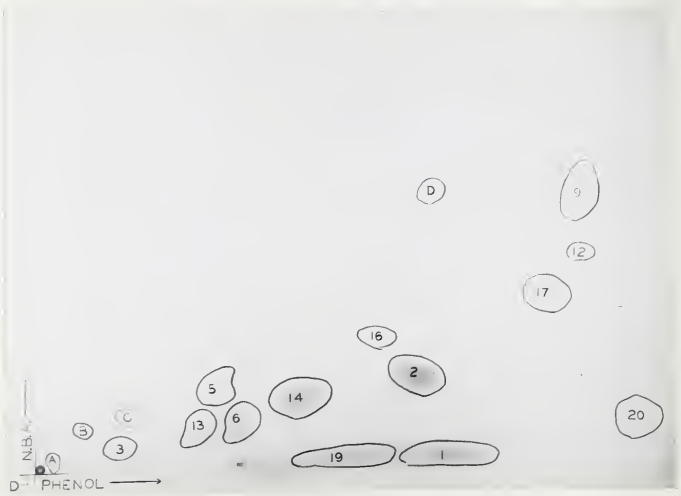


Figure 36. Chromatogram of 500 microliters of diseased wheat root extract prepared as in Figure 35.

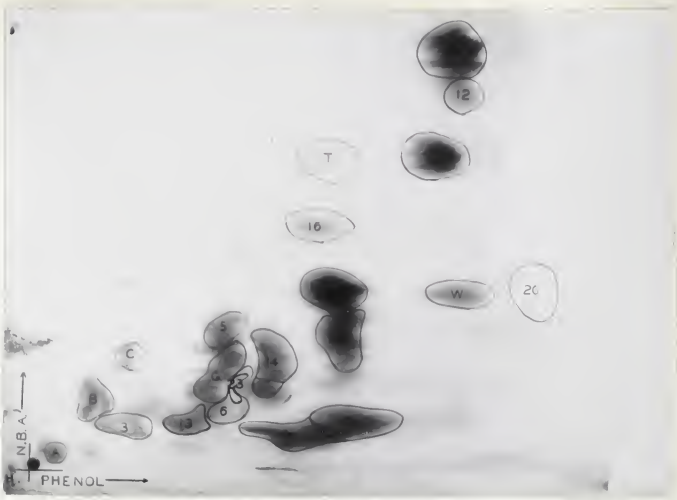


Figure 37. Chromatogram of 600 microliters of healthy wheat root extract prepared by micro hydrolysis method of 0.01 grams of dried root tissue. Solvents are N.B.A. and phenol-water.

Figure 38. Chromatogram of 600 microliters of diseased wheat root extract prepared as in Figure 37.

Figure 39. Chromatogram of 600 microliters of healthy wheat root extract prepared by micro hydrolysis method of 0.01 grams of dried root tissue. The amino acids were removed with 80% ethanol before hydrolysis. Solvents are N.B.A. and phenol-water.

Figure 40. Chromatogram of 600 microliters of diseased wheat root extract prepared as in Figure 39.

Figure 41. Chromatogram of 1000 microliters of oat roots prepared by micro extraction method of dried root sample. Solvents are N.B.A. and phenol-water.

Note: In this series of chromatograms of root extracts, the N.B.A. solvent was allowed to run off the edge of the filter paper. Therefore the position occupied by the amino acids are not true R_f values. The chromatograms of known amino acids were similarly treated (Figures 18, 19, and 19a).

From Table 4, it is seen that the amount of free and bound amino acids expressed as milligrams per gram of dry tissue is much higher in the diseased samples, viz., 111.0, as compared with 88.5 in the healthy. Kjeldahl determinations of dried root samples also showed higher values of total nitrogen in the diseased. Values:

Healthy - 60.0 milligrams N/gram dry tissue

Diseased - 79.6 milligrams N/gram dry tissue.

The results suggest that nitrogen metabolism is much higher in the diseased root tissue. Steinberg (163), in discussing amino acid action on tobacco seedlings, suggests that localization of certain amino acids may be responsible for the "frenching" symptoms. The results of the above experiment suggest that the higher concentration in the diseased root tissue of certain free amino acids as alanine, serine, asparagine and valine as well as the basic amino acids is associated with the symptoms occurring in root rot infection.

CONCLUSION AND SUMMARY

The techniques of paper chromatography discussed in Part I have been applied successfully to the analysis of amino acids in healthy and diseased wheat roots. Various extraction procedures have been discussed and it was found that analysis of dried samples using 80% ethanol as extracting fluid gave the most consistent results. With this technique the free as well as bound amino acids of healthy and diseased root tissues have been detected and quantitatively determined. In all cases there was a much higher concentration of certain amino acids in the diseased root samples and it was suggested that these amino acids were associated with the symptoms of root rot.

PART III

BIOCHEMICAL STUDIES OF THE ROOT ROT PATHOGEN *HELMINTHOSPORIUM SATIVUM*

PART III

BIOCHEMICAL STUDIES OF THE ROOT ROT PATHOGEN HELMINTHOSPORIUM SATIVUM

THE FUNGUS H. SATIVUM

According to Christensen (37), Pammel, King and Bakke, in 1910, were the first to attribute the irregular brown lesions of barley leaves to Helminthosporium sativum. In Canada the first reports of this disease appeared earlier. Simmonds (158) mentioned that Gussow reported H. sativum as occurring on barley in 1912. Christenson (37) gave a very vivid description of the symptoms of the disease produced by this fungus and mentioned that roots, leaves, stems, spikes, spikelets and seeds can become infected.

H. sativum is a member of the imperfect fungi, possessing septate branching mycelium and dark brown or black spores. These spores are septate with four to eight cells, and from each cell on germination, an infection hypha grows out that infects a plant directly or forms mycelium that is responsible for producing disease symptoms. Henry (159) found that spores were not absolutely necessary for the development of the disease and Oswald (124) mentioned that in the California area spores are very seldom found in the soil, yet the infection might be quite high. The perfect or sexual stage of this fungus

has been recently described by Tinline (174).

HISTORICAL REVIEW OF SOIL RELATIONS

Simmonds (158) has adequately reviewed the literature on root diseases published by Western Canadian workers prior to 1939. Several major approaches to the problem of common root rot are evident from this review. Sanford and Broadfoot (151) and Henry (81) initiated interest in the study of biological interrelationships. A more recent review of this "biotic antagonism" has been published by Sanford (152). Greaney and Machacek (72) and Mead (158) investigated infection as influenced by soil microflora and seed injury.

The possible role of mineral deficiencies in the root disease complex was investigated by Greaney (73), Machacek (108) and Broadfoot and Tyner (23). Tyner (177) found that infection on wheat roots grown in composts of oat straw with soil was much less than when the seedlings were grown in composts of barley or wheat straw. Several possible reasons for this difference were considered, viz., difference in microflora associated with the varied decomposition of each compost; chemical composition of the straw; presence of toxic by-products of decomposition; and the C/N ratio of the composts. The following experiment was undertaken by the present worker in an effort to isolate actual effects on pathogenicity of chemicals likely to be found in such composts. The variety of

amino acids found in hydrolyzed extracts of wheat and oat roots as compared with the few free amino acids (see chromatograms pp. 74 - 81) suggested that this group of organic compounds may play an important part in determining the variation of the virulence of this pathogen in greenhouse and field experiments as well as in general farming practice.

EXPERIMENTAL PROCEDURE

Effect of Amino Acids on Degree of Pathogenicity

Van Lanen *et al* (178) obtained attenuated strains of the tumor forming bacteria, *Agrobacterium tumefaciens*, by repeated transfers on a mannitol nitrate mineral salt medium containing in addition 0.3% glycine with the pH adjusted to 6.8. The resulting bacterial cultures failed to induce galls on punctured areas of tomato plants. Furthermore, they were able to regain virulence of this organism by repeated subculturing on a yeast medium. They found that all aliphatic amino acids as well as lysine were attenuators. The other diamino acids and the dicarboxylic acids were inactive. Braun *et al* (19), working with *Brucella abortus*, were able to get shift over from smooth to rough type of bacterial cells using alanine. With increased alanine concentration, new types of cells developed that were antigenically different from the rough or smooth type. In reviewing the literature no reference could be found on the effects of amino acids on the virulence of

fungal plant pathogens, although Christensen (38) mentioned that virulence of H. sativum was not attenuated by replanting on artificial media six times. In this laboratory an experiment was carried out to determine the effects on virulence of this fungus of 17 amino acids. The remaining amino acid, viz., proline, hydroxyproline and alpha-amino butyric acid, as well as the amines, asparagine and glutamine were not available at the time.

The earlier methods used for inoculating wheat seeds with H. sativum have been reviewed by Sallans (149), who described some eight different methods. A modification of Henry's method (81), which has been used in this laboratory for many years, was adapted. This was briefly described in Part II, see pp. 60 - 61. The Czapek's agar medium that was used had the following composition:

	Weight in grams
Sucrose	15
$NaNO_3$	2
K_2HPO_4	1
$MgSO_4.7H_2O$	0.5
KCl	0.5
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.01

The components were mixed in a liter of distilled water and then 15 grams of agar were added. This was placed in an autoclave and brought to 15 pounds pressure to melt the agar. To each 100 ml. of melted agar medium, 0.1 grams of an amino acid was added and the contents were thoroughly mixed.

This made the final concentration of amino acid one part in 1000. Except for tyrosine and the leucines, the amino acids were readily soluble in this hot agar solution. Approximately 10 ml. aliquots of this resulting medium were poured into test tubes, plugged, autoclaved for 20 minutes at 15 lbs. pressure, and finally slanted. Upon solidification of the medium (it was found that only cysteine hydrochloride prevented gelling), the tubes were inoculated with a culture of H. sativum known to be virulent. After incubation for two weeks (149) portions of the culture and the medium from the test tube were transferred to flasks, containing sterile soil; one test tube being used for six flasks thus allowing three replicates for the two types of experiments conducted. The flasks of soil were prepared as follows:

To a 3:1 mixture by volume of black loam soil and sand, 2% corn meal by weight was added. To this sufficient water was added to just prevent the soil mixture from becoming sticky. Approximately 140 grams of this mixture were added to 200 ml. flasks and sterilized at 15 lbs. pressure for four hours. After 24 hours cooling, the above fungus suspension was added. Before the test tube slant containing the culture of the fungus was divided among the six soil flasks, a portion of this culture was transferred aseptically to another test tube slant containing the same altered Czapek's medium.

Following an incubation period of 14 days, Figure 42, the contents from three of these six flasks were trans-

ferred to separate six inch pots and mixed with sufficient virgin black loam soil to fill the pot. In all, 17 amino acids were used and with Czapek's medium serving as the check, a total of 54 pots was used. After a period of two days during which time the fungus established itself in the new environment, 10 surface sterilized wheat seeds of the Red Bob variety were planted at uniform depth in each pot.

The soil inoculum of the three remaining flasks was thoroughly mixed with a sterilized iron rod and 10 surface-sterilized wheat seeds were planted directly in these flasks. the flasks were immediately replugged with the same cotton bungs, and were shaken so as to cover the wheat seeds with soil. These were allowed to grow in a temperature controlled room at 20° C whereas the pots described above were kept in the greenhouse. After a growth period of two weeks the seedlings were removed and data on germination, infection rating, stem height, and root lengths were taken. The degree of infection was rated from zero to 10 as shown in Figure 43, with a zero rating having no brown lesions on the crown region of the seedling and a 10 rating where the seedling failed to produce a stem although it may have a fair primary root system. The ratings were made on the coronal region since infection of the primary root system seemed to vary considerably with the moisture content of the soil.

The results of this experiment are shown in Table 5. They represent an average of six repeated experiments using

TABLE 5

Effect of Amino Acids on the Pathogenicity of Helminthosporium sativum to Wheat Seedlings Grown in Inoculum of the Pathogen in Flasks and Pots for Six Successive Plantings

AMINO ACIDS	FLASKS[Y]			POTS[YY]		
	Gm	Ic	St	Gm	Ic	St
DL - Alanine	88	62	7.7	92	19	22.4
L - Arginine	88	70	3.9	86	41	15.7
DL - Aspartic Acid	89	90	2.1	69	69	7.5
L - Glutamic Acid	84	91	1.9	68	74	7.4
Glycine	84	94	2.0	71	69	10.1
L - Histidine - HCl	88	62	7.9	92	26	17.2
DL - Iso-leucine	90	64	7.4	76	46	15.9
DL - Leucine	90	69	8.3	80	38	18.3
L - Lysine - HCl	87	93	2.0	68	77	6.7
DL - Methionine	92	56	10.9	91	29	18.0
DL - Nor-leucine	79	82	6.0	73	73	8.9
DL - Phenylalanine	81	94	2.4	61	68	8.3
DL - Serine	92	73	5.4	88	30	18.9
DL - Threonine	88	84	4.6	81	34	16.6
DL - Tryptophane	82	94	4.0	77	57	12.9
L - Tyrosine	81	98	1.9	73	74	8.1
DL - Valine	85	97	1.9	75	75	7.3
Check	91	93	1.9	68	77	6.7

Gm - Average percentage germination of wheat seeds.
Ic - Average percentage infection of coronal region of wheat plant.
St - Average stem height in centimeters.

[Y]Flasks - contained unmixed inoculum of H. sativum.
[YY]Pots - contained soil mixed with inoculum of H. sativum (3:1).

Analysis of Variance for Infection in Pots

Variance due to	Degrees of Freedom	Sum of Squares	Mean Squares	F	5% F	1% F
Acids (treatments)	17	132077.5	7769.3	75.21	1.70	2.11
Plantings (transfers)	5	135365.8	27073.2	262.08	2.26	3.11
Acids x Plantings	85	57767.2	679.6	6.59	1.14	1.21
Replicates	2	714.0	350.7	3.45	3.04	4.71
Error	214	22113.4	103.3			
Total	323	347323.9				

Mean significance for treatments = 6.78
Mean significance for transfers = 11.74

Figure 42. Growth in soil of H. sativum cultures transferred from Czapek's medium to which various amino acids had been added.

Top row- Left to right: nor-leucine, phenylalanine, serine, threonine, tryptophane, tyrosine, valine, Czapek's, lysine.

Bottom row- Left to right: arginine, alanine, asparatic, glutamic, glycine, histidine, iso-leucine, leucine, methionine.

Figure 43. Wheat seedlings showing varying degrees of infection. Left to right: 0-100% with 10% differences.

three replicates. Since infection ratings were from zero to 10, the percentage infection was easily computed by manipulation of the decimal point. Percentage germination was also computed in this way since 10 seeds were used. The values for stem height and number and length of roots were not tabulated.

From the above experiment with pots it may be concluded that addition of the amino acids alanine, arginine, histidine, iso-leucine, leucine, methionine, and serine to the synthetic Czapek's medium caused a marked decrease in pathogenicity of H. sativum to wheat seedlings of the Red Bobs variety. A single trial with Thatcher variety showed the same trend. The remaining nine amino acids that were used, as well as Czapek's medium alone, had little or no effect in altering the virulence.

In the flask experiment where the root rot fungus and the wheat seedling were in sterilized soil, thus eliminating the effects of other microflora, the same but less pronounced variation in pathogenicity occurred. The lowest percentage of infection was with methionine (56%) while the highest was recorded with tyrosine (98%) whereas with the pot trials the variation was from 19 to 76%. In some cases a decrease in germination of the wheat occurred, viz., on leucine, tyrosine, and phenylalanine cultures. It was rather difficult to assess the effects on stem height because of etiolation. In general, it can be stated that those tests which showed a fairly high degree of infection also showed retarded stem growth, Figures 45 and 46.

The purpose of this flask experiment was to eliminate

Figure 45. Pathogenicity to wheat seedlings of H. sativum cultured on Czapek's medium to which various amino acids had been added.

Back three rows- Left to right: alanine, asparatic, glycine, leucine, nor-leucine.

Front three rows- Left to right: arginine, glutamic, histidine, iso-leucine, methionine.

Figure 46. Pathogenicity to wheat seedlings of H. sativum cultured on Czapek's medium to which various amino acids had been added.

Back three rows- Left to right: serine, tryptophane, valine, lysine.

Front three rows- Left to right: phenylalanine, threonine, tyrosine, control.

any effects that other soil microorganisms may have on the virulence of H. sativum to wheat seedlings. Although various workers (81, 151, 202) have shown that the microbiological flora play an important role in determining the degree of pathogenicity, the above experiment has shown that chemical agents may produce the same effects. A possible explanation for this variation in fungus virulence will appear in the discussion.

EFFECT OF LIGHT ON PATHOGENICITY

Flasks of soil inoculum were prepared and incubated for 14 days as outlined in the previous section. One test tube culture was again divided among six flasks. Ten surface sterilized wheat seeds were added directly to the flasks as described above. The flasks were then divided into two groups; three were kept in a dark room at 20° C, while the other three flasks were kept in the greenhouse. After a growth period of two weeks, data on germination, infection rating, root lengths, and stem heights of the seedlings were taken. The results are shown in Table 6.

From this experiment, it appeared that neither light nor darkness have much effect on degree of pathogenicity of H. sativum, although slightly higher infection ratings were obtained in some of the flasks kept in the light. The greater stem height of seedlings in flasks kept in the dark is due to etio-

TABLE 6

Effect of Light on Pathogenicity of Helminthosporium sativum to Wheat Seedlings Grown in Flasks.

	LIGHT			DARKNESS		
AMINO ACIDS	Gm	Ic	St	Gm	Ic	St
DL - Alanine	95	45	11.7	85	56	12.8
L - Arginine	90	66	9.2	95	55	8.9
DL - Aspartic Acid	70	98	1.8	85	99	1.3
L - Glutamic Acid	85	88	2.7	80	98	2.0
Glycine	75	98	3.0	80	99	2.8
L - Histidine - HCl	90	29	12.3	90	26	18.1
DL - Iso-leucine	95	24	17.3	95	27	14.9
DL - Leucine	95	39	12.3	90	11	25.5
L - Lysine - HCl	75	97	2.1	100	49	4.9
DL - Methionine	95	3	18.2	85	2	25.4
DL - Nor-leucine	95	19	17.1	90	15	21.0
DL - Phenylalanine	95	86	3.8	90	56	11.5
DL - Serine	90	54	8.3	95	67	7.7
DL - Threonine	90	82	3.8	95	72	9.3
DL - Tryptophane	75	73	5.4	70	94	3.3
L - Tyrosine	90	87	2.8	75	99	1.7
DL - Valine	80	93	2.1	85	94	2.8
Check	80	98	2.7	80	98	2.7

NOTE: Symbols as in Table 5

lation effects and this may be partially responsible for the lowered infection ratings in the flasks kept in the dark.

EFFECT OF AMINO ACIDS ON SPORULATION

Henry (81) found that H. sativum sporulated on different soil types if they were sterilized but not in natural soil. Furthermore, addition of small amounts of unsterilized soil to the sterilized soil was sufficient to inhibit sporulation. He therefore suggested that sporulation is inhibited by saprophytic microorganisms of the soil. Simmonds et al (159) verified Henry's findings and they further mention that there are numerous conidia present on stubble and other decaying material above the ground level, and that these conidia play an important role in determination of primary infections.

An experiment was undertaken in conjunction with the pot studies described above, with the objective of determining the abundance of conidia in the soil flasks of inoculum just prior to transfer to the pots. Three portions from different parts of the surface mycelial mass (Figure 42) of the soil flask of inoculum, were removed with a dissecting needle and transferred to separate areas on a microscope slide. A drop of phenol-glycerine methyl green solution was added to them. The slides were then examined under the microscope for presence of Helminthosporium conidia. The results are shown in Table 7 and,as with the pot studies, are based on six separate experiments.

Since it was thought that the absence of spores might be responsible for the observed decrease in pathogenicity, the table was divided into two parts as shown.

TABLE 7

The Relation of Pathogenicity and Sporulation in H. sativum

HIGH PATHOGENICITY		LOW PATHOGENICITY	
AMINO ACID	SPORES	AMINO ACID	SPORES
Aspartic Acid	+	Alanine	+
Czapek's (Check)	+	Histidine	+
Glutamic Acid	+	Leucine	+
Glycine	+	Serine	+
Nor-leucine	+	Arginine	-
Phenylalanine	+	Iso-leucine	-
Tryptophane	+	Methionine	-
Tyrosine	+		
Lysine	-		
Threonine	-		

The results of this experiment indicate that there was no correlation between pathogenicity and the presence of spores. However, the observation that spores were regularily not produced by certain treatments is of interest and will require further study.

SECTION 2

Bremner (21) found that all the natural amino acids occurred in a hydrolyzed sample of neutral soil obtained from field plots. Wallace et al (183, 184) found that amino acids

Aspartic Acid	+	Alanine
Cystine & (Uracil)	+	Histidine
Glutamic Acid	+	Leucine
Glycine	+	Serine
Tyr-Leucine	+	Arginine
Isoglutamine	+	Ile-Leucine
Tryptophane	+	Isoglutamine
Tyrosine	+	
Lysine	-	
Threonine	-	

play a very important role in the nutrition of soil microorganisms especially in the rhizosphere region. The importance of this was further strengthened when Najjar (121) showed that amino acids enter bacterial cells by straight diffusion, the process being unaltered by cyanide or fluoride inhibitors and depending only on the external concentration. Virtanen and Linkola (180) found similar results with pea and clover cultures. However, their results with wheat showed that aspartic and glutamic acids were not used as a nitrogen source. In an experiment in which wheat plants were grown in sterile nutrient solution containing nitrate and $(NH_4)_2SO_4$ as well as aspartic acid, they actually found a decrease in dry weight and suggested that it was caused by an acceleration in the respiration rate. Calvin and coworkers (8, 12) have studied amino acids as carbon sources for plants by employing radioactive derivatives and analyzing the tissues by paper chromatography. The work of Wallace and Lochhead, as well as Virtanen and Linkola, suggests that the soil microorganisms and the roots of plants compete for organic nutrients such as the amino acids. The following two experiments were undertaken to determine the nutritive value of amino acids as carbon and nitrogen sources for both the wheat roots and the root rot organism.

Amino Acid as Carbon and Nitrogen Source for Plants

Czapek's medium, with the addition of an amino acid, was used as described on pp. 88 - 90. However, no sugar was added. Surface sterilized wheat seeds of the Red Bobs variety

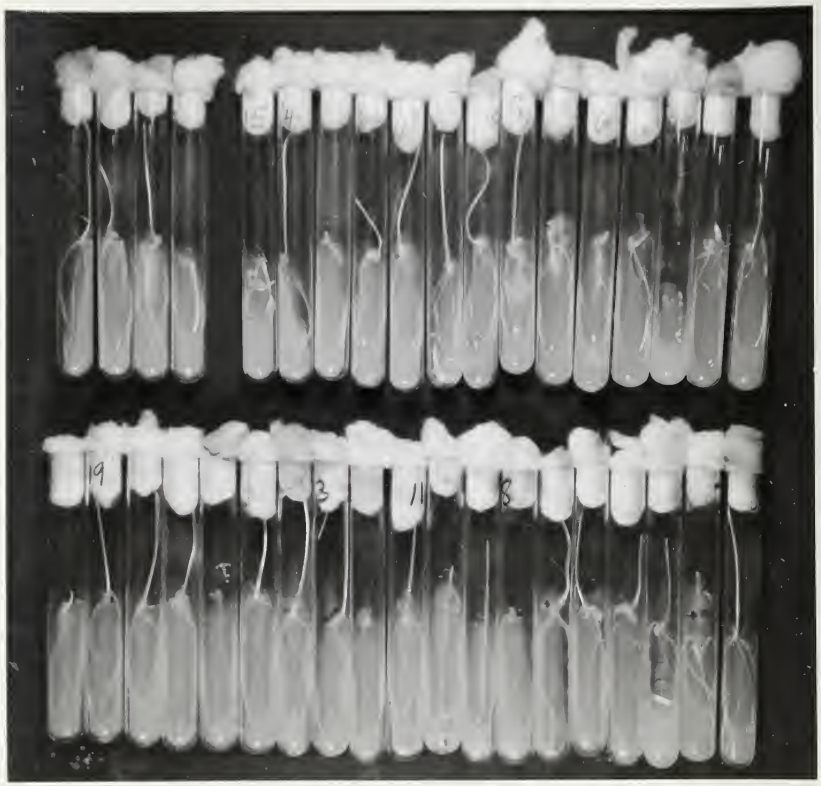


Figure 47. Effect of addition of amino acids to Czapek's medium on the growth of wheat seedlings. Sucrose omitted from the medium in the top row.

Bottom row- Left to right: proline, lysine, Czapek's, valine, tyrosine, tryptophane, threonine, serine, phenyl-alanine, nor-leucine, methionine, leucine, iso-leucine, histidine, glycine, glutamic, aspartic, alanine, arginine.

Top row- As above only tyrosine omitted.

were placed in the agar of the test tube slant by means of sterile forceps. The test tubes were placed vertically in a rack and after two weeks of growth the wheat seedlings were examined. The results are shown in Figure 47. No pronounced differences were observed and it would appear that the supply of available nitrogen and carbon in the endosperm of the wheat seed was sufficient for this two-week growth period.

Amino Acid as Carbon and Nitrogen Source for the Fungus

Although the sources of carbon and nitrogen for microorganisms in the soil are numerous, the literature contains only meagre information concerning the utilization of amino acids as carbon and nitrogen sources for growth of these microorganisms. Schultz et al (154-6) have done extensive work on the amino acids as carbon, sulphur, and nitrogen sources for different yeast strains. They found methionine, glutathione, and cysteine sulphur were not as readily used as inorganic sulphur in the form of sulphate. As carbon sources they found that if the yeast could not metabolize the carbon of glutamic acid or proline, then it was unable to utilize the carbon of any other amino acids. As a nitrogen source they conclude that amino acids fall in three groups -- unavailable, availability variable with strains of yeasts, and available. An excellent review of the nutritional requirements of fungi for carbon and nitrogen compounds is given by Steinberg (164). He pointed out that various workers have found that Aspergillus niger is capable of using a mixture of amino acids previously formed from sugar

(L-proline, L-glutamic acid, and L-ornithine), almost as efficiently as hexose. The results were almost identical with those of Schultz et al described above, who worked with yeasts. Steinberg concludes that quantitative studies of amino acid utilization must be interpreted on the basis of type of nitrogen assimilation employed by the fungus. Thus if the fungus is unable to utilize inorganic nitrogen, then it may be assumed that it uses the amino acids intact. If, on the other hand, it can use inorganic nitrogen, then deaminations probably precede utilization.

An experiment was undertaken to see if the amino acids differed in their availability as carbon and nitrogen sources for growth of H. sativum. Since nitrate and ammonium ions were present in this Czapek's medium, the fungus was therefore able to compete for these three different sources of nitrogen. In one series sucrose was included to supply the carbon sources and in the other it was omitted. The amino acids, however, were added to both series. The difference in growth after a period of 10 days is shown in Figure 48. A very fine light colored mycelial mat occurred in the test tubes without sugar in contrast to the dark heavy mass where sugar was present. As sole carbon sources it appears that proline and valine furnish the best growth. In no case was the growth equal to that which occurred when sucrose and amino acid were present. Threonine and tryptophane yielded very little growth as was also the case with the check. These results are very similar to those recorded

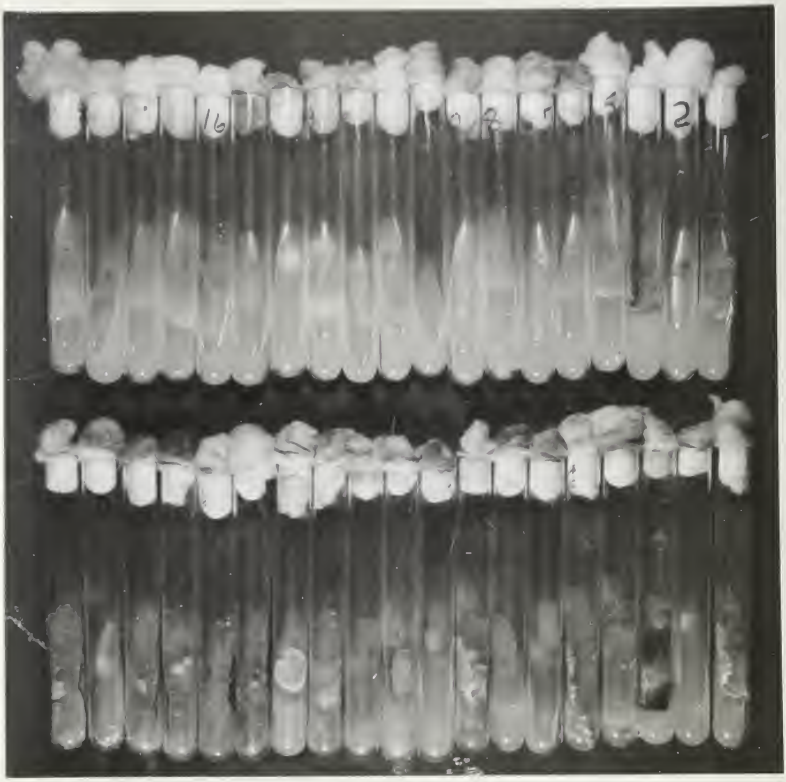


Figure 48. Effect of addition of amino acids to Czapek's medium on the growth of H. sativum. Sucrose omitted from medium in top row.

Left to right: proline, lysine, Czapek's, valine, tyrosine, tryptophane, threonine, serine, phenylalanine, nor-leucine, methionine, leucine, iso-leucine, histidine, glycine, glutamic, asparatic, alanine, arginine.

for Aspergillus niger (164). It may be concluded that all the amino acids may serve as carbon and nitrogen sources for limited vegetative growth but they do not favour sporulation at the concentration employed. Further work is necessary to eliminate the effects of NO_3 and NH_4 ions which were present in this medium, before conclusions regarding amino acids as sole source of nitrogen may be formulated.

Toxicity of Amino Acids to Plants

The degradation in the soil of nitrogenous organic residues to simple inorganic compounds includes passing through the amino acid stage. It is for this reason that the effects of amino acids on various tissues in vivo and in vitro have been studied. Riker and coworkers (66, 141) found that sunflower and tobacco tissues differed in their soil nutrient requirements as well as in the optimum concentration at which an amino acid may be applied. This difference was attributed to the amount of reserve material present in the seeds (68). In general, Riker and coworkers found that they got good to excellent growth at low and high concentrations of amino acids but inhibition at 0.001 M level which is approximately 1000 p.p.m. Isotopic studies support the view that there are three stages in a plant as regards utilization of sources of nitrogen (68). In the early stages it utilizes the N of the seed; later, when this is exhausted it must use an external source and shows preference for ammonia over amino acids, and finally when the ammonia is de-

pleted, the plant is forced to utilize amino acids. It would appear, therefore, that the toxicity of amino acids to plants would occur only in the final state. Audus and Quastel (5) found that among the amino acids only alpha-alanine and L-glutamic acid were non-inhibitors to cress roots when applied at 1000 p.p.m. Steinberg (163) found that proline and hydroxyproline could cause the "frenching" symptoms of tobacco plants in aseptic cultures and suggested that they were caused by primary localized areas of toxic concentrations of free amino acids in the tissues of the plant.

From this literature review it would appear that the high concentration of amino acids known to occur in soil as a result of degradation of nitrogenous organic compounds or as excretory products from other microorganisms (164), might be an important factor in determining the resulting symptoms of root rot. The experiment which was undertaken to determine the availability of amino acids as carbon and nitrogen sources for wheat seedlings also served to determine the toxic effects of amino acids to wheat seedlings. The concentration of amino acid used was 1000 p.p.m. The results were shown in Figure 47. Although no brown lesions (coronal) appeared, the roots showed typical lesions in most tests, especially with arginine, histidine, tryptophane, lysine and the check. With nor-leucine considerable discoloring was observed and the lateral root system appeared as knobby extrusions. In the check, although brown lesions occurred, there was an abundance of lateral roots. It is

suggested that the amino acids may be partially responsible for inhibiting lateral root development. The addition of a "corresponding" culture of H. sativum to a similar series of test tubes containing wheat seedlings, resulted in heavy root lesions even when the Czapek's medium did not contain sucrose.

From these preliminary experiments it may be tentatively concluded that certain amino acids may cause abnormal root development. Furthermore, a sugar source is not necessary for H. sativum to exert its pathogenicity to wheat seedlings.

DISCUSSION

The addition of certain amino acids to Czapek's agar medium was found to cause a decrease in virulence of the pathogen H. sativum. However, this decrease was less marked in the flask than in the pot experiments. The results of various workers who considered "biotic" antagonism as of prime importance have already been reviewed. The composition of free amino acids in root tissue was found to be a small fraction of the concentration after hydrolysis. Such tissue following decomposition in the soil should therefore provide an ample supply of free amino acids. Since free amino acids added to a synthetic medium could alter the pathogenicity of H. sativum, it is possible that a similar effect could occur in the soil. Furthermore the experiment on toxicity of amino acids to wheat seedlings grown in test

tubes on Czapek's agar medium supplemented with an amino acid, showed that certain amino acids are able to produce symptoms on roots that resemble those of root rot infection. Although the individual amino acids do not occur in the soil in the free state in as high concentrations as were used in the above experiments, their combined concentration in the soil is probably sufficient to play an important role in the expression of root rot symptoms by acting directly on the wheat seedlings or indirectly on the fungus.

CONCLUSION AND SUMMARY

The results of the above experiments suggest that certain amino acids can alter the pathogenicity of H. sativum to wheat seedlings so that in some cases infection may be completely absent. Fungus spores were not found to be essential for development of the disease. Furthermore, from the test tube experiment with wheat seedlings, it was found that a sugar source was not necessary for this fungus to cause infection although the mycelial growth was rather limited. As a carbon source the amino acids could not be used as effectively as sucrose. Since Czapek's medium contains nitrogen, the availability of amino acid as nitrogen source could not be ascertained. Certain amino acids were found to limit lateral root growth and cause necrotic areas on roots that were similar to root rot lesions. It is possible that the fungus in some way upsets the protein metabolism of the wheat root and that this causes a localization of a high concentration of different amino acids which then produce the characteristic symptoms. In turn there is probably a higher excretion of amino acids by the wheat seedling which may help to retain or alter the pathogenicity of the root rot fungus.

SUMMARY

1. The techniques employed in paper chromatography have been reviewed and successfully applied to the separation of free and bound amino acids in wheat roots.
2. For two dimensional chromatography the solvent pair N.B.A. and phenol-water (80% phenol by volume) were found satisfactory.
3. A total of 11 known amino acids and three unknown ninhydrin positive spots were found to occur in the free state in alcoholic extracts of wheat roots.
4. A total of 13 known amino acids and four unknown ninhydrin positive spots were found in alcoholic extracts of acid hydrolyzed wheat root samples.
5. The chromatograms showed that higher concentrations of amino acids, both in the free and combined form, were found in diseased wheat roots than in healthy wheat roots of the Thatcher variety.
6. Total nitrogen, determined by the Kjeldahl method was found to be higher in the diseased wheat roots than in the healthy roots.
7. Addition of the amino acids, alanine, arginine, histidine, leucine, iso-leucine, methionine and serine, singly to Czapek's medium on which the root rot fungus, H. sativum, was cultured, was found to decrease the pathogenicity of this fungus to wheat seedlings.

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